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Mendel Centenary Congress 2000 at Brno

The Mendel University of Agriculture and Forestry in Brno, together with the Plant Breeding Society (*Gesellschaft für Pflanzenzüchtung*), Germany, in cooperation with the Czech Academy of Sciences in Prague and the Mendel Genetic Society in Brno, organised on March 7–10, 2000, the *Mendel Centenary Congress* in the main pavilion at the International Exhibition Grounds at Brno. The Congress was dedicated to *100 years of genetics for plant breeding – Mendel, meiosis and marker*. Nearly 400 participants from 21 countries came to the event, that took place on the occasion of 100 years from the rediscovery of Mendel's principles of genetics.

Gregor Mendel (1822–1884) was a monk within a renown scientific community of the Augustinian monastery in Brno. Prior to becoming abbot of his monastery, he performed his famous experiments with peas. He first tested seeds of 34 pea varieties for constancy of characters in the progenies. Twenty-two of the varieties were selected for experimentation and planted annually throughout the entire experimental period. They proved very useful without exception. He grew in each of the 10 experimental years about 4–5 thousand pea plants in the monastery garden. Constancy of parental varieties was tested during the first two years and the hybrids and offsprings were evaluated in the following eight years.

Gregor Mendel presented the results of his experiments in two lectures entitled *Versuche über Pflanzen-Hybriden (Experiments with plant hybrids)*,the first on February 8th and the second on March 8th, 1865, at the *Naturforschender Verein in Brünn (Society for Natural Studies at Brno)* .

He was inspired to his experiments by artificial polinations of ornamental plants aimed to obtain new colors. In the second sentence of the introduction he stated, that "the striking regularity with which the same hybrid forms always reappeared whenever pollination between the same species took place" stimulated him to perform further experiments to study the offsprings of the hybrids. He mentioned the experiments of botanists who had crossed various forms of plants, but was aware of the nonadequacy of their methods. He also pointed to the significance of research for the developmental history of organic forms, including the enigma of generation, heredity, and fertilisation.

To study the changes of different characters in the offsprings and to find out the law for the occurrence of the characters in the progenies, he dismissed as not suitable such characters, that were difficult to distinguish, since they could be evaluated only in the sense of "more or less".

Following seven characters were selected for evaluation (the dominant characters are in bold):

1) form of ripe seeds (**round**/wrinkled), 2) colour of the seed albumen (**yellow**/green), 3) colour of the seed coat (white/**grey, brown**), 4) form of ripe pods (constricted/**non-constricted**), 5) colour of unripe pods (yellow/**green**), 6) position of flowers (**along the main axillary stem**/at the end of the stem), 7) stem length (**6–7 feet**/0.75–1.5 feet).

Only one of the two alternatives always occurred in the hybrids of the seven combinations. The characters which passed without or almost without change into the hybrids were named "dominant" and the characters which were not visible in the hybrids as "recessive". It was completely irrelevant, whether the dominant character belonged to the seed parent or to the pollen parent.

From the *Pisum* experiments Mendel explained the formation of hybrids and the segregation of progenies for the parental traits. He deduced, that a character of an offspring of a hybrid is determined by the independent random combination of two basic elements, proceeding from the original parents, and that offsprings of hybrids, in which several distinct characters have been joined, represent mathematically members of a combination array, where the segregation ratios closely match the combinatory formula.

However, the impact of the research was not understood by contemporary science. Only when chromosomes were microscopically observed, before the end of the 19th century, it was found that the segregation of characters in progenies of crosses, statistically described by Mendel, followed from the behaviour of chromosomes in the dividing cells.

The importance of Mendel's work was discovered by three scientists, who published in 1900 independently results confirming his findings. The authors were Hugo de Vries, Carl Correns and Erich Tschermak von Seysenegg. The hundredth anniversary of the rediscovery of Mendel's findings was the reason for the Congress.

The Congress was opened by the Deputy Minister of Agriculture Ing. Rybníček, the Lord-Mayor of Brno Dr. P. Duchoň, the Vice-chairman of the Japan Mendel Society Prof. T. Nagata, the Vice-chairman of the Mendel Genetic Society Prof. J. Relichová and the Rector of Mendel University of Agriculture and Forestry in Brno Prof. S. Procházka.

The opening session was dedicated to Gregor Mendel. O. Chloupek (Brno) gave a lecture about G. Mendel, P. Ruckenbauer (Vienna) about E. von Tschermak-Seysenegg , E. Zevenhuisen (Amsterdam) about Hugo de Vries and G. Röbbelen, Göttingen, about Carl Correns .

The first session was dedicated to *Mendelian genes – Principles and application in breeding*, with contributions by W. Swiecicki (Poznan), I. Panayotov (General Toshevo, Bulgaria), J. Špunar (Kroměříž, Czech Republic), A. A. Goncharenko (Nemchinovka, Russia), R. Jansen (Einbeck, Germany), W. E. Weber (Halle, Germany) and by A. E. Melchinger (Hohenheim, Germany).

The second session concentrated on *Meiosis – Mechanism and genetic control* with lectures by B. F. Chadov (Novosibirsk, Russia), D. Schweizer (Vienna), T. Lübberstedt, (Hohenheim, Germany), T. Schwarzer (Norwich, UK), I. Schubert (Gatersleben, Germany) and T. Schmidt (Kiel, Germany).

The third session dealed with *Plant reproduction – Application to breeding* and included lectures by S. Schwarz-Sommer (Cologne, Germany), G. Coupland (Norwich, UK), C. Mariani (Nijmegen, the Netherlands), T. Dresselhaus (Hamburg, Germany) and C. Glimelius (Uppsala, Sweden).

The fourth session included lectures dealing with the *The plant genome – Molecular structure and function* by R. Schmidt (Cologne, Germany), J. Matoušek (České Budějovice, Czech Republic), J. Doležel (Olomouc, Czech Republic), A. Gierl (Garching, Germany), T. Sasaki (Tsukuba, Japan) and M. D. Gale (Norwich, UK).

The fifth session was dedicated to *Non-Mendelian phenomena – Impact of apparent and real exceptions to the laws of 1865* with contributions by R. Herrman (Munich, Germany), B. Vyskot (Brno, Czech Republic), K. Tsunewaki (Matsuoka, Japan) and by H. C. Becker (Göttingen, Germany).

The Concluding Lectures dealt with the *State and future of Mendelian genetics* by F. Salamini (Cologne, Germany) and *Potentials and limitations of Mendelian genetics for breeding* by M. Frauen (Hohenlieth) and W. Friedt (Giessen, both Germany).

Contributions to the topic of the Congress were presented also on 217 posters.

The lecturers took part in a reception given by the Lord Mayor of the city of Brno. The Congress participants were invited to a sightseeing tour of the hosting city, to visit the Mendel Museum and to listen a concert (Janáček and Dvořák) at "Mendels" church on the Mendel square. During the concert break spoke P. Clemens Richter, great-grand nephew of Gregor Mendel and also Augustinian monk from Stuttgart, about his famous relative. Czech breeders invited the German colleagues to a discussion about the future of their profession. The Mendeleum – the research institute of the Mendel University, founded by Prof. Tschermak von Seysenegg at Lednice, was the target of an excursion, followed by a visit to the Moravian karst. The farewell dinner was in the revious wine cellar of the Augustinian monastery.

The participation of young scientists from the postcommunist countries on the Congress was supported by the European Union. The Congress was also supported by the *Japan Mendel Society* and by successful German and Czech breeding and seed companies.

The lectures and poster abstracts were published in *Vorträge für Pflanzenzüchtung* 2000 (the poster-abstracts in the 47th and the lectures in the 48th volume, which amount together about 600 pages). Some of the contributions, original as well as review papers, which could not be presented on the Congress due to its time limit, are published on the following pages.

O. Chloupek, Mendel University of Agriculture and Forestry in Brno

GENETIC AND MOLECULAR CHARACTERIZATION OF VIRUS RESISTANCE IN WINTER BARLEY

GENETICKÁ A MOLEKULÁRNÍ CHARAKTERISTIKA ODOLNOSTI JEČMENE OZIMÉHO K VIRŮM

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ABSTRACT: A comprehensive screening programme was carried out on approximately 2000 accessions of the Gatersleben World Collection examined for their response to *Barley mild mosaic virus* (BaMMV), *Barley yellow mosaic virus* (BaYMV-1, -2) and *Barley yellow dwarf viruses* (BYDV-PAV, -MAV). One hundred and twenty three barley accessions were resistant to all the mosaic viruses, 51 were resistant to BaMMV and BaYMV-1, 169 were BaMMV-resistant and six were resistant to BaYMV-1 and -2. Using doubled haploid populations from crosses between 5 lines resistant to BaMMV, BaYMV-1 and -2 and susceptible parents, single gene inheritance was established in crosses with HHOR 3108, Tori and Zairai-rokkaku and HHOR 1361. Two recessive genes appeared to be responsible for the resistance of HHOR 4224. To identify the resistance genes, RAPD and microsatellite markers linked to the resistance loci *rym4* and *rym5* were used. Fourteen accessions from the examined collection were moderately or highly tolerant to BYDV. Studies with a PCR-based marker linked to the gene *Ryd2* indicated that their tolerance was conferred by gene(s) different from *Ryd2*. The BYDV tolerance of the cultivar Post was investigated using a doubled haploid population of the cross Post/Rubina// HHOR 3108. This study indicated that genes with minor effects might be responsible for tolerance of this cultivar to BYDV.

Keywords: *Hordeum vulgare* L.; *Barley mild mosaic virus; Barley yellow mosaic virus; Barley yellow dwarf virus*; resistance; tolerance; PCR-markers

ABSTRAKT: Přibližně u 2000 vzorků ječmene ze Světové kolekce v Gaterslebenu byla podrobně sledována reakce vůči viru mírné mozaiky ječmene (*Barley mild mosaic virus* – BaMMV), viru žluté mozaiky ječmene (*Barley yellow mosaic virus* – BaYMV-1, BaYMV-2) a virům žluté zakrslosti ječmene (*Barley yellow dwarf viruses* – BYDV-PAV a BYDV-MAV). Ze sledovaných vzorků ječmene bylo 123 odolných vůči všem mozaikovým virům, 51 k BaMMV a BaYMV-1, 169 k BaMMV a 6 k BaYMV-1 i k BaYMV-2. Použitím dihaploidních populací z křížení mezi 5 liniemi odolnými k BaMMV, BaYMV-1 a BaYMV-2 a náchylnými rodiči byla zjištěna monogenní dědičnost odolnosti v kříženích s HHOR 3108, Tori a Zairai-rokkaku a HHOR 1361. Dva recesivní geny podmiňovaly rezistenci HHOR 4224. K identifikaci genů rezistence byly použity RAPD a mikrosatelitní markery ve vazbě s lokusy rezistence *rym4* a *rym5*. Čtrnáct vzorků zkoumané kolekce bylo mírně nebo vysoce tolerantních k BYDV. Studie s markerem založeným na PCR ve vazbě s genem *Ryd2* poukazovala na toleranci na odlišném základě než na genu *Ryd2*. Tolerance odrůdy Post vůči BYDV byla zkoumána pomocí dihaploidní populace z křížení Post/Rubina//HHOR 3108. Výsledky poukazovaly na možnost, že tolerance této odrůdy k BYDV je podmíněna minorgeny.

Klíčová slova: *Hordeum vulgare* L.; *Barley mild mosaic virus*; *Barley yellow mosaic virus*; *Barley yellow dwarf virus*; odolnost; tolerance; PCR-markery

INTRODUCTION

The viruses of the barley yellow mosaic virus complex (*Barley mild mosaic virus* – BaMMV, *Barley yellow mosaic virus* – BaYMV-1 and -2), the *Barley yellow dwarf viruses* (BYDV-PAV and MAV) and *Cereal yellow dwarf virus* (CYDV-RPV) are the cause of the most important virus diseases of winter barley in Europe. The cultivation of resistant cultivars is the only practical way of controlling the barley yellow mosaic viruses. Out of the 63 winter barley cultivars registered in Germany, 28 were found resistant to BaMMV and BaYMV-1 (Anonymous, 1999).

Apart from the completely resistant cultivar Tokyo with resistance gene *rym5*, the resistance of the other cultivars is based on gene *rym4*. This gene confers resistance to BaMMV and BaYMV-1.

In contrast to the mosaic viruses the resistance situation concerning BYDV is different. There are no virus-resistant or tolerant cultivars registered in Germany. Of the two known resistance genes *ryd1* and *Ryd2* (Suneson, 1955; Schaller *et al.*, 1964) only the more effective gene *Ryd2* has been used in barley breeding. The British spring barley cultivar Coracle (Catherall *et al.*, 1977) and the winter barley cultivars Vixen (Parry and Habgood, 1986), Wysor (Starling *et al.*, 1987) and Venus (Brown *et al.*, 1988) are known to carry this resistance gene. The effectiveness of the *Ryd2* gene depends, however, on the genetic background (Jones and Catherall, 1970), the tested virus (Baltenberger *et al.*, 1987) and the environmental conditions (Schaller, 1984). The genetic constitution of other winter barley genotypes, described as 'moderately resistant or tolerant' to BYDV is unknown, although polygenic inheritance has been suggested.

The importance of the above mentioned cereal viruses started to increase in Germany in early 1980s. For these reasons we initiated a research programme into these viruses, which included the examination of the winter barley collection of the genebank at the Institute for Plant Genetics and Crop Plant Research, Gatersleben.

MATERIAL AND METHODS

About 2000 accessions were screened for their reaction to different viruses. To study the inheritance of virus resistance or tolerance, doubled haploid (DH) populations were produced from crosses between resistant accessions and susceptible parents using the *bulbosum* technique (Kasha and Kao, 1970; Pickering and Devaux, 1992). BaYMV resistance tests were carried out on naturally infested fields, artificially layed out plots and by mechanical inoculation in a climate chamber. All plants were serologically analysed with DAS-ELISA or direct tissue blot immunoassay to determine the presence and levels of virus in leaf tissue.

BYDV tests were performed in the field and relied on naturally occurring infections to enable the identification of accessions that showed either weak symptoms or no visible signs of infection. These barley accessions and a population of 125 doubled haploid lines were inoculated with viruliferous aphids (*Rhopalosiphum padi*, *Sitobion avenae*) in field and glasshouse tests (using BYDV-PAV and -MAV). The symptom expression, plant height, num-

Table 1. Resistance/susceptibility of DH lines derived from crosses between accessions resistant to BaMMV(M)/BaYMV-1(Y1) and/or BaYMV-2(Y2) and susceptible parents

Cross	Viruses tested	Number of lines			χ^2 (1:1)	Conclusion
		tested	resistant	susceptible		
[DH 1/25 (Post × Rubina) x HHOR 3108]*	M/Y1/Y2	174	80	94	1.126	1 gene
HHOR 3158 × Tori	M/Y1	95	49	46	0.095	1 gene
Zairai-rokkaku × HHOR 3158	M/Y1/Y2	30	13	17	0.533	1 gene
	M/Y1	66	35	31	0.242	1 gene
HHOR 1361 × HHOR 3158	M/Y1/Y2	20	7	13	1.800	1 gene (?)
HHOR 10714 × HHOR 4224	M/Y1/Y2	59	16	43	0.212**	2 genes

**Barley yellow mosaic virus* resistant parents are underlined; ** χ^2 (1:3)

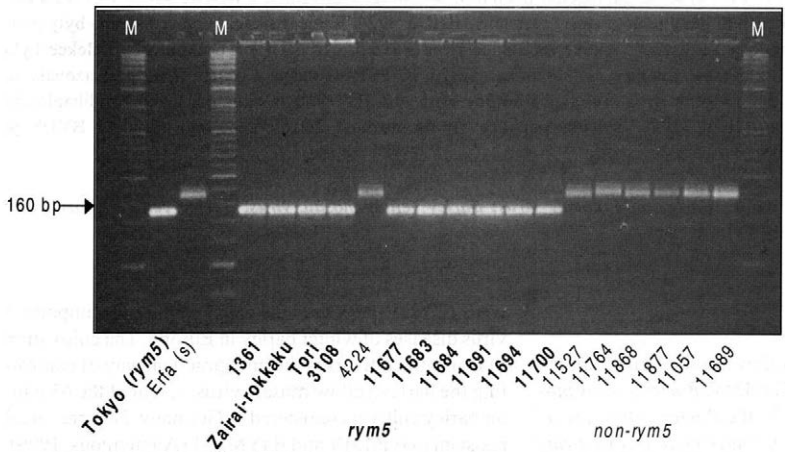

Fig. 1. Detection of the gene *rym5* in winter barley accessions with complete resistance to BaMMV, BaYMV-1 and BaYMV-2 using the microsatellite marker BMac29

ber of ears/plant, grain yield/plant and thousand kernel weight were the examined traits.

Genomic barley DNA was prepared as described by Graner *et al.* (1991). PCR amplifications, using the microsatellite BMac29, which is linked to *rym5*, were carried out in a reaction volume of 12,5 µl according to the protocol of Graner *et al.* (1999). The *rym4* gene was detected using the linked RAPD marker OP-Z04A (Schiemann *et al.*, 1997). PCR reactions (12,5 µl) were carried out using the marker *Ylp* for identifying the *Ryd2* allele as developed by Ford *et al.* (1998).

RESULTS AND DISCUSSION

In the tests of resistance to the mosaic viruses, 123 accessions appeared to be completely resistant, 51 were resistant to BaMMV and BaYMV-1, 169 were resistant to BaMMV, and six were resistant to BaYMV-1 and -2 (Proeseler *et al.*, 1999). Among 14 BYDV-tolerant barley accessions (Habekuß and Proeseler, 1996) there were detected accessions that possess combined resistance to mosaic viruses and *Drechslera teres* (Proeseler *et al.*, 1999).

Inheritance studies using DH populations derived from crosses between five mosaic-resistant accessions and susceptible parents, indicated the presence of a single resistance gene in four accessions, whereas the fifth accession (HHOR 4224) appeared to contain two recessive genes (Table 1). Using the microsatellite marker BMac29 (Graner *et al.*, 1999) linked to *rym5*, four accessions, that showed segregation for one resistance gene, appeared to contain the gene *rym5*, whereas HHOR 4224 did not (Fig. 1).

145 accessions resistant to mosaic viruses were analysed using PCR-markers very closely linked to the resistance genes, respectively the RAPD marker OP-Z04A (Schiemann *et al.*, 1997) for the gene *rym4* and the microsatellite marker BMac29 for the gene *rym5* (Graner *et al.*, 1999) (Table 2). In 15 of the 22 accessions that were resistant to BaMMV and BaYMV-1, a fragment of 640 bp was amplified, which is known to be associated with *rym4* in Ragusa (Fig. 2). After analysing 123 accessions resistant to BaYMV complex with BMac29, 104 samples showed the resistance conferred by *rym5* connected with the presence of a 160 bp amplified fragment known to be associated with the resistance of the variety Tokyo (Fig. 1).

Further inheritance studies and analyses using molecular markers are undoubtedly needed with the barley materials that showed a complete resistance to the mosaic viruses and which do not contain *rym5*.

Table 2. Detection of *rym4* or *rym5* in winter barleys with resistance to the mosaic virus complex by using the molecular markers OP-Z04A or BMac29 linked to *rym4* and *rym5*, respectively

Resistance to	Number of accessions			Characteristics of non-*rym4* or *rym5* genotypes
	tested	*rym4*	*rym5*	
BaMMV+BaYMV-1	22	15	0	*rym5**, *rym8* or unknown gene
BaMMV+BaYMV-1+BaYMV-2	123	0	104	*Rym2*, *rym6*, *rym11* or unknown gene

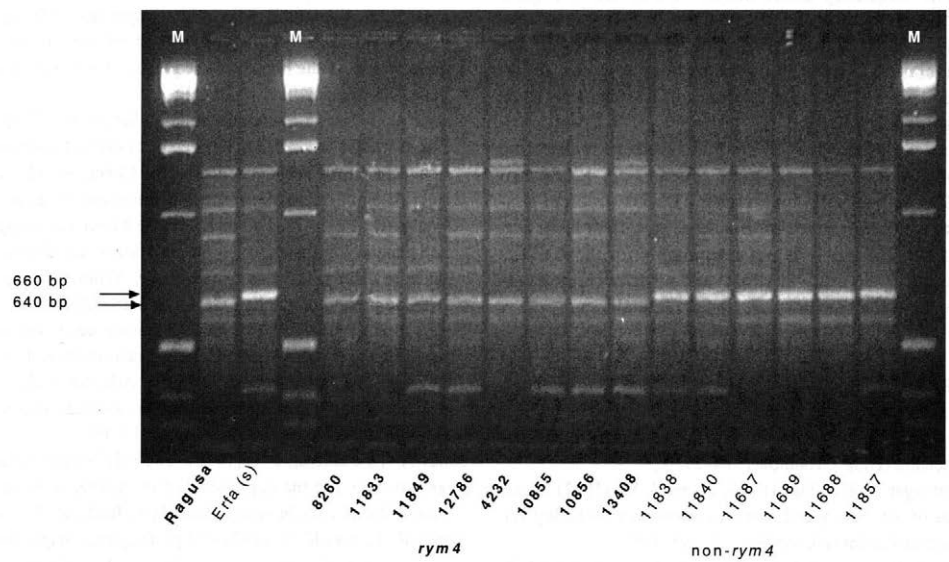

Fig. 2. Detection of the *rym4* gene in different winter barley accessions using the RAPD marker OP-Z04A (Ragusa = resistant control)

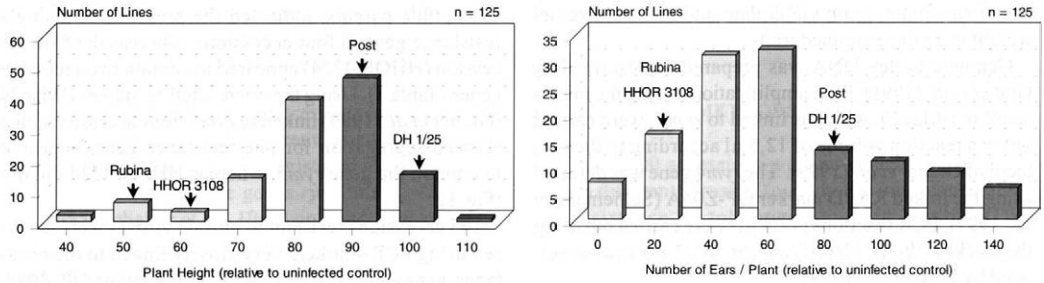

Fig. 3. Plant height and numbers of ears/plant of doubled haploid lines of the cross DH 1/25* × HHOR 3108 after artificial BYDV-PAV inoculation compared with uninfected controls in the field 1998/99 [* BYDV-tolerant line of the cross (Post × Rubina)]

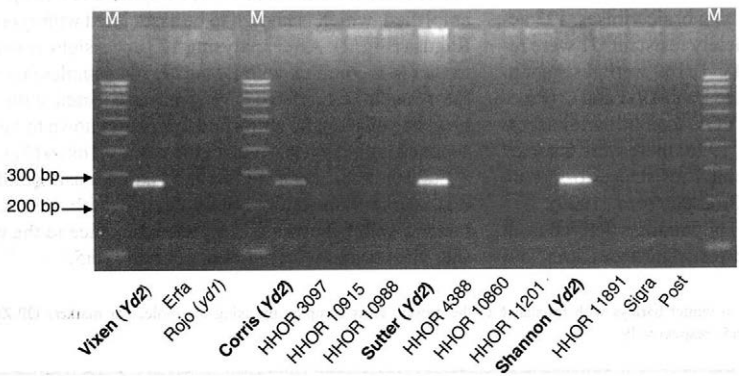

Fig. 4. Detection of the gene *Ryd2* in BYDV-tolerant barley accessions using the linked allele-specific PCR-marker at the *Ylp*-locus

The DH population of the cross DH 1/25 × HHOR 3108 was used to study the inheritance of BYDV-tolerance of DH 1/25, a high yielding line from the cross Post × Rubina. The frequency distributions of the lines for plant height and number of ears per plant showed a continuous variation and there was no clear segregation in different classes (Fig. 3). This can be interpreted as further evidence that the BYDV-tolerance of Post is likely to be polygenically inherited.

The development of the *Ylp* PCR marker for detecting the presence of the *Ryd2* gene (Ford *et al.*, 1998) provides us with a useful tool to check BYDV-tolerant genotypes for the presence of this gene. It is clear from Fig. 4 that the *Ryd2* gene is not responsible for the BYDV-tolerance of the cultivar ‚Post' and other accessions selected from the barley collection at Gatersleben.

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RESISTANCE TO THE BARLEY YELLOW MOSAIC VIRUS COMPLEX – FROM MENDELIAN GENETICS TOWARDS MAP BASED CLONING*

ODOLNOST VŮČI KOMPLEXU VIRŮ ŽLUTÉ MOZAIKY JEČMENE – OD MENDELOVSKÉ GENETIKY KE KLONOVÁNÍ NA BÁZI MAPOVÁNÍ

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ABSTRACT: Soil-borne barley yellow mosaic virus disease caused by *Barley mild mosaic virus* (BaMMV), *Barley yellow mosaic virus* (BaYMV) and BaYMV-2 has gained evident importance in most European barley growing countries today. By Mendelian segregation analyses different recessive resistance genes have been identified within the barley gene pool and integrated into the RFLP-map of barley. Based on PCR-based markers different strategies for combining these genes ("pyramiding") are conducted and a map based cloning approach for the isolation of the *Rym5*-locus conferring resistance to BaMMV, BaYMV and BaYMV-2 using RAPDs and AFLPs for high resolution mapping is in progress. The present state of this research is briefly reviewed.

Keywords: barley (*Hordeum vulgare*); barley yellow mosaic virus disease (BaMMV, BaYMV, BaYMV-2); molecular markers; pyramiding; high-resolution mapping

ABSTRAKT: Ve většině evropských zemí pěstujících ječmen se vážnou chorobou staly půdou přenášené virové žluté mozaiky ječmene, jejímiž původci jsou virus mírné mozaiky ječmene (BaMMV) a viry žluté moziky ječmene (BaYMV a BaYMV-2. Analýzou mendelovského štěpení byly v genovém základu ječmene identifikovány různé recesivní geny rezistence a byly integrovány do RFLP-mapy ječmene. Na základě markerů založených na PCR jsou používány různé strategie pro kombinace těchto genů (pyramiding) a probíhají práce na izolaci lokusu *Rym5*, zajišťující rezistenci k BaMMV, BaYMV a BaYMV-2, při využití technik RAPD a AFLP pro mapování na vysoké rozlišovací úrovni. Práce podává stručný přehled současného stavu tohoto výzkumu.

Klíčová slova: ječmen (*Hordeum vulgare*); viry žluté mozaiky ječmene (BaMMV, BaYMV, BaYMV-2); molekulární markery; pyramiding; mapování na vysoké rozlišovací úrovni

Barley yellow mosaic virus disease – caused by a complex of at least three soil-borne viruses, i.e., BaMMV, BaYMV and BaYMV-2 (Huth, 1989) – has become a major threat to winter barley cultivation in Europe due to a constant spread and high yield losses frequently observed in susceptible winter barley crop. Because of transmission by the soil-borne fungus *Polymyxa graminis* chemical measures against the disease are neither efficient nor economic. However, based on Mendelian segregation analyses and tests for allelism it was shown that at least against BaMMV different recessive resistance genes are present within the barley gene pool (Ordon and Friedt, 1993; Götz and Friedt, 1993). Some of these genes have already been integrated into the RFLP-map of barley on chromosomes 3HL, 4HL, 5HS and 1HS, respectively (Graner and Bauer, 1993; Graner *et al.*, 1995, 1999a; Bauer *et al.*, 1997; Saeki *et al.*, 1999) and PCR-based markers have been developed for *rym4*, *rym5*, *rym9* and *rym11* (Ordon *et al.*, 1995, 1999). These markers are well suited for marker assisted selection, enhanced backcrossing procedures and "pyramiding" of respective resistance genes – a task which cannot be solved without marker techniques but may prevent the selection of new virus strains as reported from Japan (Kashiwazaki and Hibino, 1995) and thereby leading to longer lasting resistances.

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Attempts to combine different resistance genes in one breeding line ("pyramiding") were carried out using different strategies exclusively based on DH-lines due to the fact that DHs are homozygous and respective recessive genotypes are more frequent than in F_2-populations. On the one hand F_1-progenies of single crosses (e.g., *rym4* × *rym9*, *rym9* × *rym11*) were used for DH-line production and plants being homozygous recessive at both loci were identified by the respective PCR-based markers. These genotypes were crossed again followed by DH-line production and marker based identification of DHs being homozygous for *rym4*, *rym9* and *rym11*. On the other hand, F_1 plants [e.g., *(rym4* × *rym9)* × *(rym4* × *rym11)*] were inter-crossed and out of about 100 kernels those being homozygous at one resistance locus and heterozygous at the others were identified by markers (6.25%) and used for DH-line production in a next step, leading to an offspring of 25% having three resistance genes and 50% having two genes fixed homozygous recessively (Fig. 1).

Besides these applications in barley breeding closely linked molecular markers may serve as a starting point for the isolation of respective resistance genes *via* map

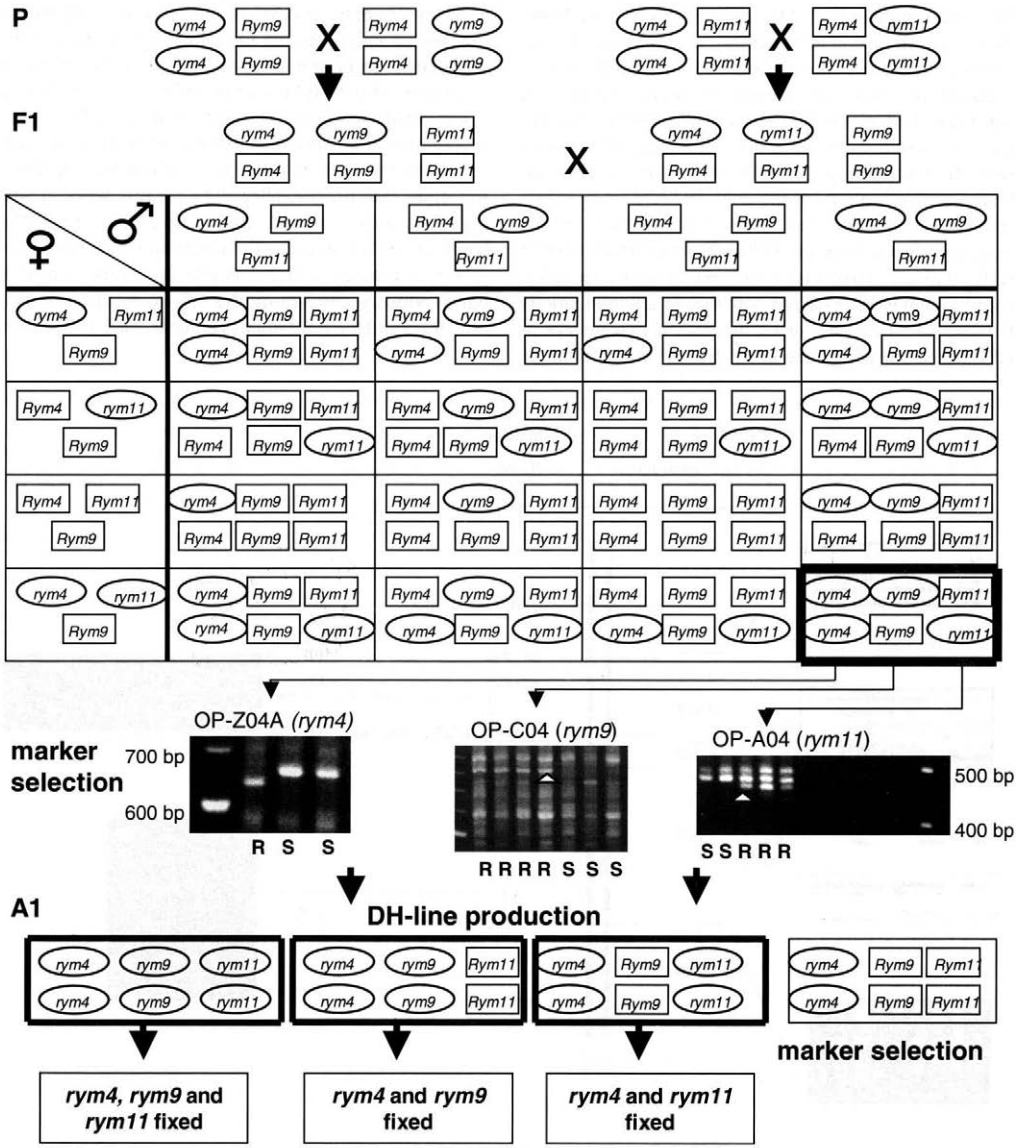

Fig. 1. Pyramiding of resistance genes *rym4*, *rym9*, and *rym11* using one haploid step

based cloning (cf. Büschges *et al.*, 1997). In European barley breeding *rym4* conferring resistance to BaMMV and BaYMV and *rym5* encoding additionally resistance to BaYMV-2 – which form a complex locus in the telomeric region of chromosome 3HL (Graner *et al.*, 1999b) – are of special importance due to their wide application. However, up to now nothing is known about the structure and function of these genes and it is still an open question whether this locus on chromosome 3HL consists of several closely linked genes or the different resistance specifities are the result of multiple allelism. In order to bridge the gap between the genetic map established on recombination [centiMorgan (cM)] and the physical map [(bp), for review cf. Ordon *et al.*, 2000], a map based cloning approach has been chosen to isolate the *Rym5*-locus.

Based on two codominant markers (MWG838, MWG010/BMac029; cf. Graner *et al.*, 1999a) flanking *rym5* in a distance of 0.8 and 1.3 cM, respectively, a high-resolution mapping population was constructed by analyzing 1,026 F_2-plants of the cross W122/37.1 (*rym5*) × Alraune resulting in a resolution of 0.049 cM. F_2-plants carrying a recombination within the target interval were selfed and in F_3 10 plants of each heterozygous recombinant F_2 plant were re-tested with these flanking markers to identify homozygous recombinants which were repeatedly tested for resistance in F_4. Based on this strategy the *Rym5*-locus was mapped about 1.16 cM proximal of MWG010 and 0.35 cM distal to MWG838. For marker saturation of the respective region bulked segregant analyses using 1,200 RAPDs and 1,536 fluorescence detected *Eco*RI/*Mse*I AFLP primer combinations were conducted. Up to now, one RAPD- and 7 AFLP-markers were mapped within this interval with the closest mapping 0.049 cM proximal and 0.880 cM distal of *rym5*. The closest linked AFLP (0.049 cM) has already been converted into an STS-marker which amplifies a single fragment specific for chromosome 3HL as revealed by a set of wheat barley addition lines (Fig. 2). This STS is currently used for screening a BAC-library. Future work will be focused on enlarging the mapping population and further marker saturation followed by STS development in order to construct a BAC-contig bridging the *Rym5*-locus.

The results obtained elucidate that using Mendelian segregation analysis as a starting point molecular markers on the one hand facilitate the implementation of new strategies into practical breeding for resistance against the barley yellow mosaic virus complex and on the other hand are useful tools for the isolation of the respective genes; a strategy which has proven its usefulness in barley already concerning the *Mlo*-locus (Büschges *et al.*, 1997) and the *Rar1*-locus (Lahaye *et al.*, 1998).

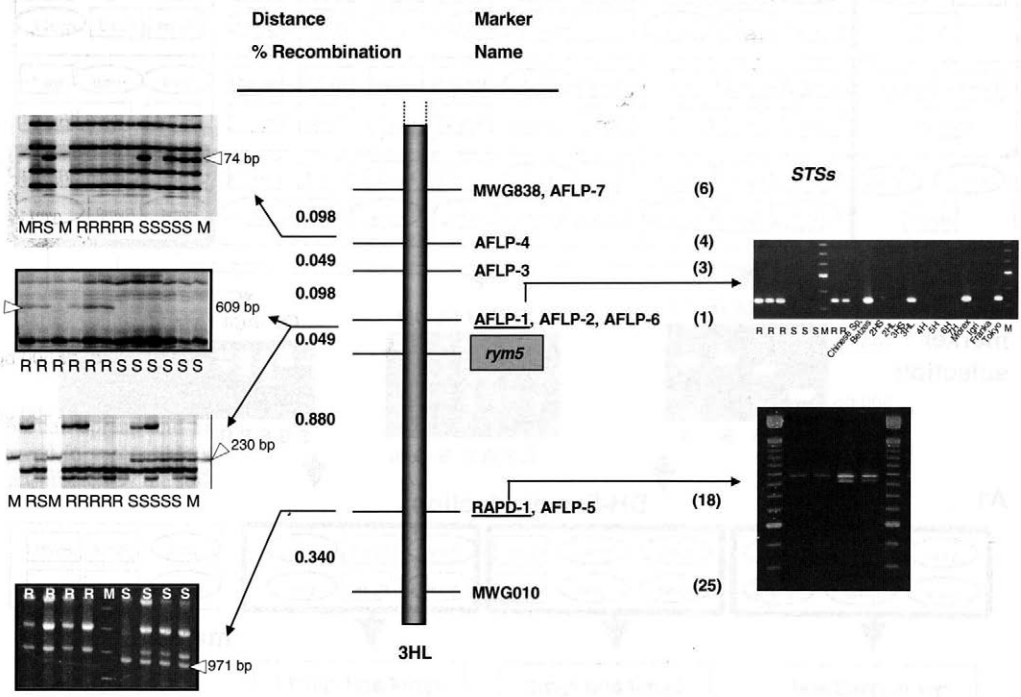

Fig. 2. High resolution map of the *Rym5*-locus based on the analysis of 1026 F_2-plants of the cross W122/37 (*rym5*) × Alraune

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THE RESISTANCE OF BARLEY TO LEAF STRIPE CAUSED BY *PYRENOPHORA GRAMINEA*

ODOLNOST JEČMENE K PRUHOVITOSTI VYVOLANÉ HOUBOU *PYRENOPHORA GRAMINEA*

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ABSTRACT: Leaf stripe is a seed-borne barley disease, caused by *Pyrenophora graminea*. Reactions of cultivars to leaf stripe show great variability, and one race-specific resistance system (*Rdg1a* gene) has been described. In this work it was studied the response of seven two-rowed and two six-rowed cultivars, and of a two-rowed line to the highly virulent isolates *Dg2* and *Dg5* of *Pyrenophora graminea*. NILs have been developed from two highly resistant six-rowed winter cultivars, Thibaut and Onice, after six backcrosses to susceptible cultivar Mirco, in order to map these new sources of resistance by means of BSA (Bulked Segregant Analysis). Colinear maps of barley chromosome 1(7H) have been drawn, where the major QTL of resistance of cv. Proctor to leaf stripe and two other resistance (R) genes to barley pathogens have been mapped. These R genes, *RsmMx* to the seed-borne virus BSMV (Barley Stripe Mosaic Virus), and *Rpt4* to *Pyrenophora teres*, are associated to comon markers in the barley genome and could be two candidates to explain the biological role of the leaf stripe QTL.

Keywords: leaf stripe; *Pyrenophora graminea*; barley; resistance; QTLs

ABSTRAKT: Pruhovitost ječmene je semeny přenášená choroba, kterou vyvolává *Pyrenophora graminea*. Reakce odrůd na pruhovitost je velmi variabilní a je popsán systém rasové specificity (gen *Rdg1a*). V práci je popsáno chování sedmi dvouřadých a dvou šestiřadých ječmenů a dvouřadé linie vůči velmi virulentním izolátům *Dg2* a *Dg5*. Ze dvou vysoce rezistentních šestiřadých ozimých odrůd (Thibaut a Onice) byly po šesti zpětných kříženích s náchylnou odrůdou Micro vytvořeny blízce izogenní linie (NIL), aby bylo možné pomocí BSA (ramšové analýzy štěpení) zmapovat tyto nové zdroje rezistence. Byly vytvořeny kolineární mapy chromozomu 1 ječmene (7H), na němž byl lokalizován hlavní QTL rezistence odrůdy Proctor a dva další geny (R) pro rezistenci. Tyto geny rezistence, *RsmMx* vůči viru čárkové mozaiky ječmene přenášenému semeny, a *Rpt4* vůči *Pyrenophora teres*, by mohly být v důsledku společné lokalizace v genomu ječmene klíčem k vysvětlení biologické role QTL pro pruhovitost.

Klíčová slova: pruhovitost listů; *Pyrenophora graminea*; ječmen; rezistence; QTL

INTRODUCTION

Leaf stripe is a one year-cycled seed-borne disease specific of barley, caused by *Pyrenophora graminea* (Ito and Kuribayashi) anamorph (*Drechslera graminea* [Rabenh. ex. Schlech.] Shoemaker). Soil temperatures below 12°C during seed germination favour the infection of the rootlets and after this event the fungus colonises the plants systemically. Therefore, leaf stripe is a serious barley disease especially where cold weather conditions occur in the early stages of plant growth, as in Scandinavian (spring sowing) and Mediterranean (winter sowing) barley cultivation areas, causing reductions of grain yield (Porta-Puglia *et al.*, 1986). Reactions of barley cultivars to the disease show great variability, ranging from complete susceptibility to complete resistance, and also race-specific resistance systems have been demonstrated. For example cv. Golf is resistant to five European and one Japanese fungal isolates, but not to the Syrian isolate *Sy-1* (Thomsen *et al.*, 1997). Cultivar Thibaut is resistant to the Italian isolate *Dg2*, and susceptible to the isolate *Dg5* (Gatti *et al.*, 1992). The resistance behaviour of seven two-rowed and two six-rowed cultivars and of two-rowed line to the leaf stripe isolates *Dg2* and *Dg5* is reported in Table 1. The degree of genotype resistance was evaluated as percentage of diseased plants after artificial infection of the germinating seeds. The inoculation has been performed by the "sandwich method", where 120 barley seeds for each genotype are put to germinate at cold (6°C) between two layers of the fungal mycelium in active growth. The cultivars Georgie and Golf descending from Vada, showed a similar resistance level as their ancestor when tested with the two isolates; the same can be observed for Maris Otter, descendant of Proctor. The six-rowed variety Passport proved to be totally susceptible to leaf stripe, and for this reason it is proposed to be used as a check in artificial inoculations as well as the Afghani line CI6944. The highly resistant cultivar Rebelle

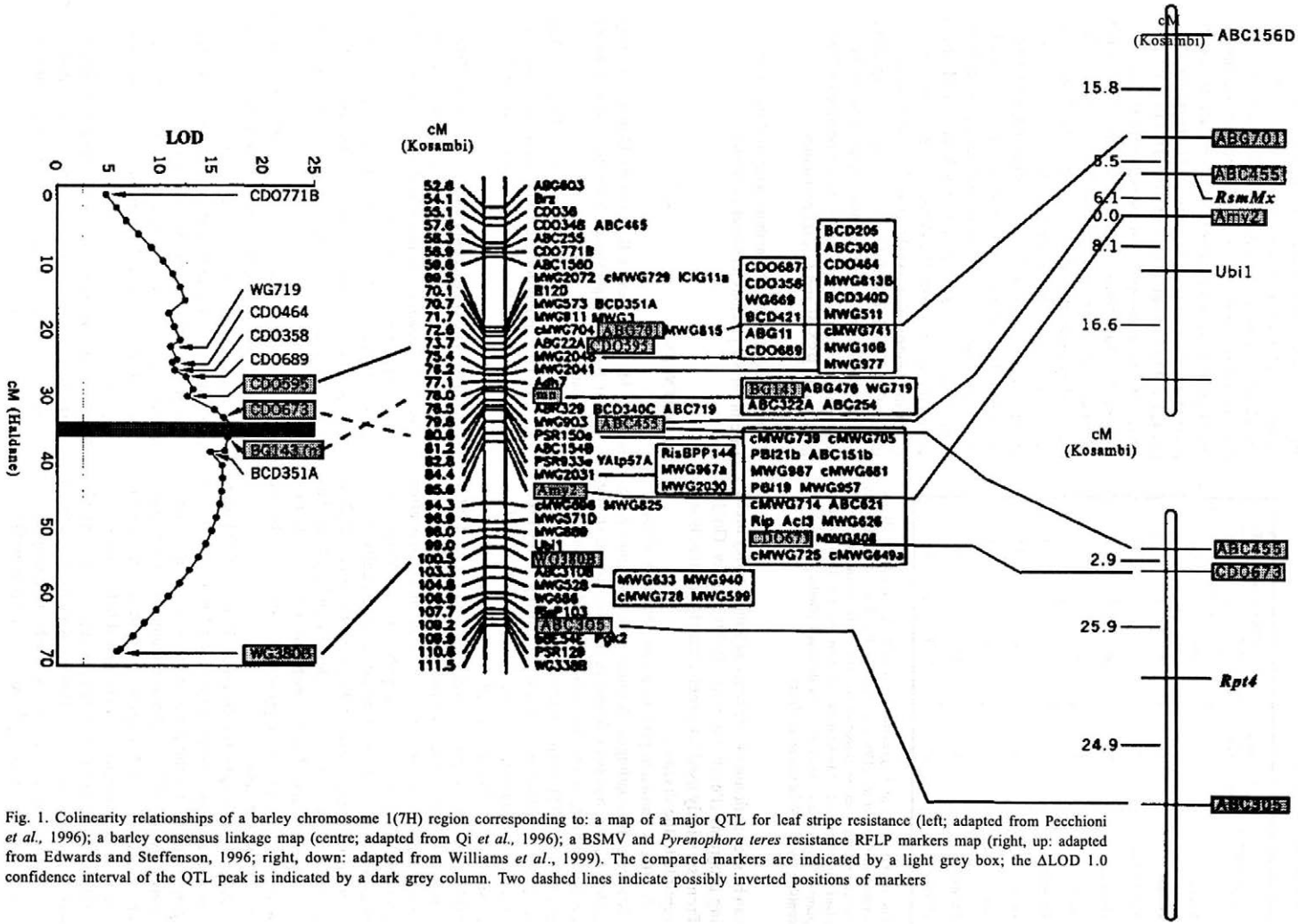

Fig. 1. Colinearity relationships of a barley chromosome 1(7H) region corresponding to: a map of a major QTL for leaf stripe resistance (left; adapted from Pecchioni *et al.*, 1996); a barley consensus linkage map (centre; adapted from Qi *et al.*, 1996); a BSMV and *Pyrenophora teres* resistance RFLP markers map (right, up: adapted from Edwards and Steffenson, 1996; right, down: adapted from Williams *et al.*, 1999). The compared markers are indicated by a light grey box; the ΔLOD 1.0 confidence interval of the QTL peak is indicated by a dark grey column. Two dashed lines indicate possibly inverted positions of markers

Table1. Reactions to barley leaf stripe isolates *Dg2* and *Dg5* of seven two-rowed and two six-rowed cultivars and of a two-rowed line after artificial inoculaton using the "sandwich method"

Barley cultivar	Two-/Six-rowed (T)/(S)	Resistance to isolate (%)	
		Dg2	*Dg5*
Rebelle	S	0.0	0.0
Proctor	T	12.0	6.0
Maris Otter	T	6.4	0.0
Golden Promise	T	39.4	31.3
Vada	T	6.0	9.0
Georgie	T	10.0	3.1
Golf	T	3.0	5.5
Alf	T	5.4	9.5
Passport	S	100.0	100.0
CI6944	T	94.0	96.0

The highly virulent *P. graminea* isolates *Dg2* and *Dg5* are described in Gatti *et al.* (1992). Following Pecchioni *et al.* (1999), barley cultivars can be classified as 'Highly resistant' when <5% plants are diseased; 'Moderately resistant' with 5–15% diseased plants; 'Susceptible' with 15–40% diseased plants; 'Highly susceptible' with >40% diseased plants

can be used as donor of resistance in winter barley breeding programs (Pecchioni *et al.*, 1999), and cv. Golden Promise, widely used for genetic transformation, is susceptible to the disease.

A little is known at present about genetic basis of resistance to this pathogen. A qualitative resistance gene, called *Rdg1a*, has been found in the two-rowed spring cv. Alf and mapped to chromosome 2(2H) (Thomsen *et al.*, 1997). A QTL with a major effect on resistance level has been identified in the two-rowed spring barley Proctor and mapped to the centromeric region of barley chromosome 1 (7H) (Pecchioni *et al.*, 1996). Three other minor QTLs have been mapped as well (Pecchioni *et al.*, 1996).

Recently, NILs have been prepared from highly resistant six-rowed winter cultivars Thibaut and Onice, after six backcrosses with the susceptible cultivar Mirco. The resistance gene of cv. Thibaut is specifically active against the highly virulent *P. graminea* isolate *Dg2*; a RAPD analysis is currently underway and could lead to the identification of polymorphisms associated to this new source of resistance present in a six-rowed barley genetic background.

After the first QTL results on cv. Proctor, it had been our aim to map several barley PR (Pathogen Related) genes induced by the pathogen to verify map associations between QTLs and these candidate genes (Pecchioni *et al.*, 1999). No significant map associations had been found between the loci responsible for PRs and the QTLs of resistance to leaf stripe, suggesting the hypothesis that other genes or, following Robertson (1985), a less effective allele of an R gene can be the biological determinant of the major QTL. In this direction, in Fig. 1 it can be observed the colinearity of the maps of barley chromosome 1(7H) where the major effect QTL and two other resistance genes have been mapped. These R genes, *RsmMx* of resistance to the seed-borne virus BSMV (Barley Stripe Mosaic Virus) (Edwards and Steffenson, 1996), and *Rpt4* of resistance to the fungus *Pyrenophora teres* (Williams *et al.*, 1999), are associated to common markers (Fig. 1) and could be two candidates to explain the biological role of the QTL of cv. Proctor. Alternatively in this centromeric region of barley chromosome 1, a clustering of different resistance factors may have occurred. The resistant allele of *RsmMx* is carried by the cv. Morex, and that of *Rpt4* by the cv. Galleon, that is derived from cv. Proctor. Doubled haploids (DH) mapping populations and molecular marker maps have been developed from the crosses between cultivars Steptoe and Morex, and between Galleon and Haruna Nijo. These populations are available and they have already been scored for resistance to BSMV and to *P. teres* respectively for the mapping of *RsmMx* and *Rpt4*. Different reactions of parental material to leaf stripe were observed and populations of DH lines will be used to verify cosegregation and co-mapping of the resistance to *P. graminea* with BSMV and *P. teres* resistance.

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GENETIC BASIS OF CEREAL CROPS BREEDING FOR APHID RESISTANCE*

GENETICKÝ ZÁKLAD ŠLECHTĚNÍ OBILNIN NA REZISTENCI VŮČI MŠICÍM

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ABSTRACT: Differential interactions based on major genes for resistance were shown between genotypes of the English grain aphid (*Sitobion avenae*) and wheat, of the bird cherry-oat aphid (*Rhopalosiphum padi*) and wheat and of the greenbug (*Schizaphis graminum*) and sorghum. Moreover, differential interactions between weakly expressed minor resistance genes in sorghum and greenbug genotypes were found. Consequently, aphid adaptation does not depend on the phenotypic expression and genetic control of resistance. Resistant varieties with various genetic basis should be cultivated. The possibilities to increase the genetic diversity (use of cultivated forms, introgression, mutagenesis) depends on crop features. The gene pools of soft and durum wheat are poor in aphid resistant forms but only cultivated sorghum species have very effective greenbug resistance. At least 7 new genes for greenbug resistance were identified in sorghum. Our experiments also revealed that somaclonal variation can be used to increase genetic diversity for greenbug resistance in wheat and barley.

Keywords: cereal crops; aphids; genetics of resistance

ABSTRAKT: V práci jsou popsány differenciální interakce založené na majorgenech pro rezistenci mezi genotypy mšice *Sitobion avenae* a pšenice, mšice *Rhopalosiphum padi* a pšenice a mšice *Schizaphis graminum* a prosa. Dále byly nalezeny diferenciální interakce mezi sotva zřetelnými minorgeny rezistence prosa a genotypy této mšice. Z toho vyplývá, že adaptace mšic je nezávislá na fenotypické expresi a genetické kontrole rezistence. Měly by se pěstovat rezistentní odrůdy s rozdílným genetickým základem. Možnosti zvýšení genetické diverzity (používání pěstovaných odrůd, introgrese, mutace) závisí na vlastnostech plodiny. Genové základy pšenice měkké i pšenice tvrdé jsou chudé na formy odolné k mšicím. Pouze pěstované druhy prosa mají velmi účinnou odolnost vůči mšicím. Nejméně 7 nových genů odolnosti vůči mšicím bylo identifikováno v prosu. Pokusy prokázaly, že somaklonální variace je využitelná pro zvýšení genetické diverzity odolnosti vůči mšicím u pšenice a ječmene.

Klíčová slova: obilniny; mšice; genetika rezistence

INTRODUCTION

Aphids are serious economic insect pests of cereal crops. Host plant resistance is an ideal pest management strategy. However, resistant varieties may be short-lived as new insect biotypes overcome them. It is therefore necessary to broaden the genetic diversity for aphid resistance in cereals.

Resistance in *Triticum* ssp. to the most abundant and harmful pests for wheat in Russia, the bird cherry-oat aphid (*Rhopalosiphum padi* L.) and the English grain aphid (*Sitobion avenae* F.), was studied in different regions of the former SU.

Of 4527 durum and soft wheat samples only 48 showed very low levels of infestation by aphids under field conditions. Some of the selected varieties possess the antibiosis (the adverse effect of resistant plants on aphid biology) against *R. padi*. The samples Delfi 400 (k-54046, Kazakhstan) and ELS (k-43578, Norway) proved to be the most resistant. Delfi 400 showed antixenosis (the non-preference of aphids for plants under free choice) to some populations of *R. padi*. Genetic studies showed that the resistance in variety Delfi 400 is conditioned by one dominant and one recessive genes. This cultivar is highly resistant to some *R. padi* populations, but analysis of Daghestan population indicated the occurrence of biotypes that overcome the resistance of Delfi 400.

One method of minimizing damage to wheat is to identify and use resistant wild *Triticum* germplasm in interspecific crosses with cultivated wheat. In total, 1043 germplasms, representing 30 rare *Triticum* species were screened for aphid resistance. Analysis of polyploid wheats showed a considerable variability among wild species in resistance to *S. avenae* and *R. padi*. The most

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promising sources for aphids resistance are species with the genomes A^u (*T. urartu*), A^b (*T. boeoticum*, *T. monococcum*) and A^bGD (*T. kiharae*, *T. miguschovae*). These species show high level of antibiosis and antixenosis. The resistance in wheats of the *Timopheevii* section might be overcome. For instance, based on field and laboratory experiments differential interaction of *S. avenae* with *T. zhukovskyi* (A^bA^bG genome) was discovered. The species became fully susceptible to the Daghestan population of the pest. At the same time it is a highly resistant to the Usbek and North-Western populations of *S. avenae*. Some level of resistance was shown in *T. timopheevii* (A^bG genome), but at very high aphid density this species failed to retain the resistance. Natural aphid populations appear to be highly polymorphic and virulent biotypes are accumulated over one period of vegetation.

Heavy infestation of some samples by *S. avenae* is combined with high resistance to *R. padi* – in other words, plant resistance to these aphids is conditioned by different genes. *T. kiharae*, *T. miguschovae* and some *T. monococcum* samples were found to be highly resistant to both aphid species.

The greenbug, *Schizaphis graminum* Rond., is a key pest of sorghum. In Russia, a Hungarian variety Sarvasi has been involved in crosses since the 1960s as a single donor of resistance. Field and laboratory trials show that Sarvasi and the donors of resistance employed in the USA are ineffective in Russia. Resistance to greenbug was studied in 5059 samples and 25 highly resistant lines were isolated. Only cultivated forms had very effective resistance.

We have found differential interactions of sorghum genotypes with the pest populations. Obviously, European and Asian greenbug populations are not connected. An analysis of greenbug clones, sampled from sorghum fields solely at the Cuban Experimental Station of VIR (Krasnodarskii krai), revealed a wide variability in host plant exploitation by the pest. It has been possible to classify the aphid into at least 10 phenotypes of virulence, or biotypes (Table 1).

Usually the lines with noneffective genes have a low level of resistance. This weakly expressed resistance to virulent greenbug clones is controlled by minor genes, independent or slightly linked with major genes of resistance. They interact differentially – with the aphid and, contrary to Van der Plank's (1968) postulates, they can not be a basis of durable (horizontal) resistance. Obviously, there does not exist a connection between the degree of expression, the genetic control of resistance and the stability of resistance. The possibility of rapid overcoming of minor genes for greenbug resistance was confirmed by observations of seasonal dynamics of the structure of the natural aphid population on the variety Sarvasi.

We identified 11 genes for greenbug resistance in sorghum (Table 2). The sample k-457 (PI 264453, USA) has one dominant (*Sgr1*) and one recessive (*Sgr2*) genes for resistance. Gene *Sgr1* was identified in samples i-589430 (PI 264453, Spain) and k-3852 (Sarvasi, Hungary). We suppose that these accessions have also gene *Sgr2*. Samples k-9921 (Shallu, USA) and k-9922 (KS-30, USA) have in common the incompletely dominant gene for resistance *Sgr3*. The gene symbol *Sgr4* was assigned to the dominant gene controlling greenbug resistance in k-6694 (Deer, USA). The resistance of k-1362 (Durra Belaya, Syria) is determined by one dominant (*Sgr5*) and one recessive (*Sgr6*) genes. The variety Sorgogradskoe (k-9436, Rostovskaya oblast) and the accession k-1240 (Dzhugara Belaya, China) carry the gene *Sgr5*. Samples k-1362 and k-1240 seem to share a recessive gene for resistance. The sample k-924 (Dzhugara Belaya, China) has one dominant (*Sgr7*) and one recessive (*Sgr8*) genes. At least one of these genes is present in k-923 (Dzhugara Belaya, China). Resistance in k-930 (Dzhugara Belaya, China) is controlled by two dominant complementary genes (*Sgr9*, *Sgr10*). One of the two dominant genes in the accession k-1237 (Dzhugara Belaya, China) is designated by the symbol *Sgr11*.

The genes *Sgr5–Sgr11* were not used in breeding and, with the exception of *Sgr6*, they are effective against the Krasnodar pest population. The genes *Sgr1–Sgr4* and *Sgr6* are effective against some clones from the Krasnodar greenbug population. The genes in k-1362 and k-9436 are effective only against the European greenbug populations; while those of Sarvasi confer resistance only to Asian populations.

Results of our investigations showed that "own" or alien genes for resistance can lose their effectiveness due to genetic adaptation in pests. We studied, if the use of nontraditional mutagenesis, e.g., induction of soma-

Table 1. Resistance of sorghum samples to different virulence phenotypes of *S. graminum* (from the Krasdodar greenbug population, 1993–1998)

Sample	# of greenbug virulence phenotype									
	1	2	3	4	5	6	7	8	9	10
Sarvasi	R	R	**R**	**R**	S	S	S	S	**R**	S
Deer	R	R	S	S	S	S	**R**	S	S	S
Shallu	R	S	S	S	S	R	S	S	S	S
Sorgogradskoe	R	R	R	S	R	R	R	S	S	S
Durra Belaya	R	R	R	**R**	R	R	R	**R**	S	S

R – resistant, S – susceptible

Table 2. Genes for greenbug resistance in sorghum

Gene symbol	Mode of inheritance	Tester lines	Efficiency
Sgr1	dominant	k-457, k-3852, i-589430	low
Sgr2	recessive	k-457, k-3852 (?), i-589430 (?)	low
Sgr3	dominant	k-9921, k-9922	low
Sgr4	dominant	k-6694	low
Sgr5	dominant	k-9436, k-1362, k-1240	high
Sgr6	recessive	k-1362, k-1240	low
Sgr7	dominant	k-924, k-923 (?)	high
Sgr8	recessive	k-924, k-923 (?)	high
Sgr9, Sgr10	dominant complementary	k-930	high
Sgr11	dominant	k-1237	high

clonal variation, can result in selection of resistant genotypes. As a model we used the systems barley – *S. graminum* and wheat – *S. graminum*. More than 1500 R_2 lines in the first system and 1200 lines in the second were assessed for resistance to the Krasnodar population of the pest and some segregating lines were isolated. Levels of the resistance expression were low, but significantly higher than in the initial varieties.

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GENETIC ANALYSIS OF FLAG LEAF ANGLE IN WINTER WHEAT

GENETICKÁ ANALÝZA ÚHLU PRAPORCOVÉHO LISTU PŠENICE OZIMÉ

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ABSTRACT: The inheritance of the flag leaf angle at the heading stage in wheat was studied in a 4 × 4 half diallel. ANOVA for combining ability showed the presence of additive and non-additive gene effects. Additive gene action appeared to be particularly important in determining this character. The line Erect Raf E-2, showing the highest negative GCA effect, was the best combiner for erect flag leaf position.

Keywords: winter wheat; flag leaf angle; half diallel; combining ability analysis

ABSTRAKT: Dědičnost úhlu praporcového listu pšenice ve stadiu metání byla studována v polovičním dialelním pokusu 4 × 4. ANOVA pro kombinační schopnost vykázala přítomnost aditivních i neaditivních genových efektů. Aditivní genové efekty se jevily obzvlášť důležité pro determinaci této vlastnosti. Linie Erect Raf E-2, vykazující nejvyšší negativní všeobecnou kombinační schopnost (GCA), byla nejlepším přenašečem vzpřímeného postavení praporcového listu.

Klíčová slova: pšenice ozimá; úhel praporcového listu; poloviční dialel; analýza kombinační schopnosti

INTRODUCTION

Reduction of plant height and increase in stand density of the wheat crop resulted in an increased interest of researches in the leaf architecture and its effect on the productivity of photosynthesis and hence on grain yield. The flag leaf position has been widely used as a characteristics in cultivars descriptions (Siddique and Perry, 1990). Borojević and Kraljević-Balalić (1984), have reported that the angle of leaf insertion is a variable character that could be selected for. Some of the experimental materials showed the erect leaves when the crop was in the heading stage, but horizontal ones during the grain filling stage. Fifteen percent of wheat cultivars (1400 cultivars coming from 36 countries were included in experiments) had erect upper leaves before anthesis, and half of these materials showed horizontal or pendulous position at milk grain stage (Borojević and Denčić, 1984).

Positive effects of an erect leaf position yield of grain in wheat were reported by Hsu and Walton (1970) and Chatta (1992). Conversely, Carvalho and Qualset (1978), Borojević and Kraljević-Balalić (1983), and Araus *et al.* (1993) did not find any advantage of erect leaves even in dense stands.

The aim of this study was to get information about the inheritance of the flag leaf angle in the generations F_1 and F_2 of wheat diallel crosses.

MATERIAL AND METHODS

Four winter wheat cultivars (lines) were included in a half diallel crossing scheme. Erect Raf E-2, from Mexico and UC-65680, from USA, represented genotypes with erect leaves; Bezostaya-1, from former USSR and Sava from Yugoslavia, represented genotypes with curved leaves. Parents and hybrids of the F_1 and F_2 generations were grown at the experimental field at Rimski Šančevi, Yugoslavia. The experiment was conducted in a randomized block design in three replications. The plots were 2.5 m^2, 100 cm long, the row to row distance was 20 cm, and the plant to plant distance 10 cm. The angle of flag leaf (FLAN), formed by the stem and the lamina, was measured with a protractor; at the heading stage. Thirty randomly selected plants of the parents and F_1 hybrids and ninety F_2 plants of were analysed in each cross.

The analysis of combining ability was performed according to Griffing (1956): method 2, model I.

RESULTS AND DISCUSSION

Table 1 showed that the parents and hybrids in both generations differed singnificantly in the mean flag leaf angle.

Table 1. Mean values for flag leaf angle (°) in parents and hybrids of F_1 and F_2 generations

Parents	(1)	(2)	(3)	(4)
1. Erect Raf E-2	**14.6**	16.8	17.5	19.3
2. UC-65680	17.3	**17.3**	22.5	23.6
3. Sava	21.8	22.3	**22.5**	19.2
4. Bezostaya-1	17.4	22.4	21.0	**19.5**

$LSD_{0.05}$ (F_1) 0.97 (F_2) 0.89
$LSD_{0.01}$ 1.33 1.22
*above the diagonal F_1, under the diagonal F_2 values

The variation in both the general (GCA) and specific (SCA) combining ability for flag leaf angle in F_1 and F_2 was found to be highly significant, indicating that genes with additive and non-additive effects were involved in the expression of this character (Table 2). Our results are in agreement with those of Christaldo *et al.* (1992). They reported that both the additive and dominance gene effects were important in the expression of erect and pendent leaves. On the contrary, Dhindsa *et al.* (1992) and Simon (1999) reported that only additive gene effects were responsible for the flag leaf angle of wheat.

Table 2. ANOVA for combining ability in flag leaf angle

Source of variation	DF	Mean squares	
		F_1	F_2
GCA	3	14.98**	38.25**
SCA	6	4.94**	16.22**
error	18	0.11	0.09
GCA/SCA		3.03	2.36

The line Erect Raf E-2 exhibited significant negative values of GCA i both generations. It is the best general combiner for erect leaf position. The cultivar Sava had highly positive GCA effect and showed good combining ability for curved leaf position (Table 3).

Table 3. GCA values for the flag leaf angle in wheat

Parent GCA	F_1	R*	F_2	R*
1. Erect Raf E-2	–2.269	1	–2.025	1
2. UC-65680	0.181	2	–0.225	2
3. Sava	1.297	3	1.969	3
4. Bezostaya-1	0.792	4	0.281	4
SE (g_i)	0.115		0.106	

* rank order

It is useful to compare combining ability values of the parents with their mean performance for the flag leaf angle. It can be deduced from the data that close association between the GCA and the mean performance of the parents was present, which indicates that parents may be selected on the basis of their mean values.

The cross Erect Raf E-2 × Sava was found to be the cross with highly significant SCA value for erect leaf position on the basis of both examined generations (Table 4).

Table 4. SCA values for flag leaf angle in wheat

Crosses	SCA	
	F_1	F_2
Erect Raf E-2 × UC-65680	–0.394	–0.020
Erect Raf E-2 × Sava	–0.811	–1.153
Erect Raf E-2 × Bezostaya-1	1.494	–0.426
UC-65680 × Sava	1.739	0.886
UC-65680 × Bezostaya-1	3.334	2.774
Sava × Bezostaya-1	0.792	2.286
SE (S_{ij})	0.206	0.190

Concerning the leaf position a possible strategy could be to select genotypes that have erect upper leaves before anthesis, but during grain filling (when lower leaves are quickly senescing) switch the position of leaves to a more horizontal form. The joint efforts of physiologists, geneticists and plant breeders may result in development of alternative selection criteria helpful for further yield improvement (Slafer and Andrade, 1991).

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BIOCHEMICALLY ESTIMATED SEGREGATION OF THE ANTHER CULTURE-DERIVED PROGENY OF A WINTER WHEAT F_1 HYBRID WITH A HETEROZYGOUS 5DL.5DS/5DL.5RS CHROMOSOME PAIR

BIOCHEMICKÁ ANALÝZA ŠTĚPENÍ POTOMSTVA PRAŠNÍKOVÉ KULTURY F_1-HYBRIDU PŠENICE OZIMÉ S HETEROZYGOTNÍM PÁREM CHROMOZOMŮ 5DL.5DS/5DL.5RS

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ABSTRACT: DH 5841, a doubled haploid wheat line carrying a 5DL.5RS-translocation chromosome besides the 1BL.1RS-chromosome, was crossed to wheat cultivar Amadeus 7143, the Amadeus-subline with a 1BL.1RS-translocation. The resulting F_1 hybrid with a heterozygous 5DL.5DS/5DL.5RS chromosome pair was subjected to haploid technique. From the plantlets regenerated in anther culture, a total of 57 lines was available as dh_2-generation. These lines were identified regarding the constitution of chromosome 5D. The presence of the long wheat chromosome arm 5DL was confirmed by means of the patterns of NADP-dependent aromatic alcohol dehydrogenase. As markers for the short chromosome arms 5DS or 5RS, the pertinent isoenzymes of shikimate dehydrogenase were used. In this way, 30 dh_2-lines were determined to possess a 'normal' 5DL.5DS chromosome, and 27 lines the 5DL.5RS-translocation. The ratio of 30:27 approximately agrees with the theoretically expected 1:1-segregation.

Keywords: wheat-rye-translocation; biochemical marker; anther culture; doubled haploid lines; segregation; Mendelian laws

ABSTRAKT: DH 5841, dihaploidní linie pšenice obsahující mimo chromozom 1BL.1RS translokovaný chromozom 5DL.5RS byla křížena s linií pšenice Amadeus 7143, což je sublinie odrůdy Amadeus s translokací 1BL.1RS. Ze vzniklého F_1hybridu s heterozygotním párem chromozomů 5DL.5DS/5DL.5RS bylo haploidní technikou z regenerovaných rostlinek z prašníkových kultur získáno celkem 52 linií generace dh_2. U těchto linií byla zjišťována konstituce chromozomu 5D. Přítomnost dlouhého ramene chromozomu 5DL byla zjišťována pomocí vzorku dehydrogenáz aromatického alkoholu závislé na NADP. Jako markery krátkého ramene chromozomu 5DS nebo 5RS byly použity odpovídající izoenzymy dehydrogenázy šikimátu. Tímto způsobem bylo určeno 30 dh_2 linií obsahujících ‚normální'chromosom 5DL.5DS a 27 linií s translokací 5DL.5RS. Poměr 30 : 27 přibližně odpovídá teoreticky očekávanému štěpení v poměru 1 : 1.

Klíčová slova: pšenično-žitná translokace; biochemický marker; prašníková kultura; dihaploidní linie; štěpení; Mendelovy zákony

INTRODUCTION

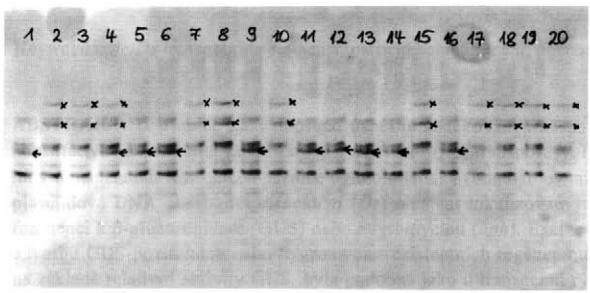
The agronomical success of the wheats with a 1BL.1RS-rye-translocation has suggested the transfer of further rye chromosomes, such as 4R or 5R, into the wheat genome. Therefore,dh 5841 (a doubled haploid wheat line carrying a 5DL.5RS-translocation chromosome besides the 1BL.1RS-chromosome, Vahl and Müller, unpublished) has been crossed to the wheat cultivarAmadeus 7143 (the Amadeus-subline with a 1BL.1RS-translocation). The resulting F_1-hybrid IJ 98 with a heterozygous 5DL.5DS/5DL.5RS chromosome pair has been subjected to haploid technique using the anther culture method according to Müller and Vahl (1993). After a successful colchicin-treatment of the haploid plantlets and two seed-sets of the resulting doubled haploids, 57 lines are available as dh_2-generation. Seedlings of these lines are used for biochemical estimation of anther culture-derived progeny regarding the constitution of the chromosome 5D (5DL.5DS or 5DL.5RS). According to Vahl and Müller (1997), shikimate dehydrogenases (SKDH) and a NADP-dependent aromatic alcohol dehydrogenase (AADH) are suitable markers for the detection of 5DS or 5RS, and for 5DL, respectively.

For the isoenzyme analyses, single leaflets are homogenized in the appropriate extraction medium (1:10 = w/v) with mortar and pestle. For IEF, samples are prepared with distilled water, for PAGE with 0.1 M Tris-HCl-buffer pH 7.5 containing 0.005 M EDTA, 0.04 M 2-mercaptoethanol, 0.1 M KCl and 0.5 M sucrose. SKDH is separated by IEF in 0.5 mm thin-layer gels with 5% acrylamide, 0.15% BIS, 4% Servalyte carrier ampholytes (pH 4–5/pH 4–7/pH 3–10 = 1:1:1) in horizontal DESAPHOR HF chambers. Electrode wicks are soaked in cathode fluid pH 10 and anode fluid pH 3 (SERVA), respectively. After prefocusing (1 h at 5 W), the samples are loaded 20 mm from the cathode by means of an applicator strip (20 µl extract per slot). Then, the gels are focused at 12 W for 6000 Vh, and for further 30 min. at 18 W. The isoenzymes of AADH are separated by PAGE in a modification of the gel system No. 6 of Maurer (1971) with running gels pH 7.5 (15% acryl-amide, 0.125% BIS) and stacking gels pH 5.7 (5% acryl-amide, 1.25% BIS). Electrophoresis is performed in 155 mm wide and 2 mm thick vertical gels (70 mm effective separating distance) with anodic direction (electrode buffer: Tris-Glycine pH 8.3) at a constant current of 20 mA for about 3 hrs. Sample slots formed by inserting combs into the stacking gel are loaded with 50 µl extract. After a pre-incubation in the appropriate buffer for 15 min, the gels are stained by means of the following solutions for SKDH: 30 mg shikimic acid, 15 mg NADP, 30 mg MTT, 4 mg PMS in 100 ml 0.1 M Tris-HCl- buffer pH 8.5, respectively for AADH: 180 mg cinnamyl alcohol, 15 mg NADP, 40 mg JNT, 2 mg PMS in 100 ml 0.1 M Tris-HCl-buffer pH 8.8.

On the example of the parents dh 5841 (with the 5DL.5RS-translocation) and Amadeus 7143 (with a 'normal' 5DL.5DS chromosome), Fig. 1 demonstrates the way, the short chromosome arms 5RS and 5DS are detected through the occurrence of the appropriate shikimate dehydrogenases-isoenzymes, encoded by the genes *Skdh-R1* and *Skdh-D1*, located in 5RS and 5DS, respectively. In the same manner, the anther culture progeny derived from IJ 98 = A. 7143 × dh 5841 has been classificated. The SKDH zymograms of some dh-lines are also given in Fig. 1. According to the constitution of the crossing parents, all dh-lines produced from IJ 98 should possess the long wheat chromosome arm 5DL. For some dh-lines this is confirmed, showing the presence of the 5DL-encoded NADP-dependent aromatic alcohol dehydrogenase isoenzyme (Fig. 2). In this way, altogether 30 dh_2-lines from IJ 98 were determined to carry a 'normal' 5DL.5DS-chromosome. In the other 27 lines, the 5DL.5RS-translocation was detected.

Although the number of dh-lines studied hitherto was not immense, it is already evident that the ratio of 30:27 between the pollen-derived lines without and with a *5RS*-rye-translocation nearly agrees with a 1:1-segregation. This is supported by the χ^2-test, resulting in a χ^2-value of 0,07 (χ^2crit. = 3.84, $P = 0.05$).

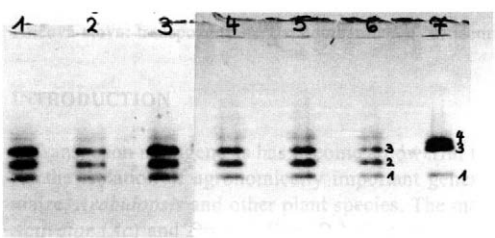
In accordance to the Mendelian laws, a diploid F_1-hybrid which is heterozygous for one special trait (here:



Water soluble extracts from single leaves; separation by IEF in the pH-gradient 4-5/4-7/3-10; – = cathode, + = anode

control of the SKDH-bands: x = *Skdh-R1* (located in 5RS), ← = *Skdh-D1* (located in 5DS)

Fig. 1. Classification of doubled haploid wheat lines (lanes 1–9 and 12–20) from F_1-population IJ 98 = Amadeus 7143 × dh 5841 regarding the presence of the short chromosome arms 5DS and/ or 5RS by means of shikimate dehydrogenase isoenzymes in comparison to their crossing parents dh 5841 (lane 10) and Amadeus 7143 (lane 11)



Buffer soluble extracts from single leaves; separation by PAGE pH 7.5; – = cathode, + = anode; for comparison in lane 1: Amadeus 7143 (with a 5DL.5DS-chromosome), lane 6: dh 5841 (with a 5DL.5RS-translocation), lane 7: L123 (our starting line with a 5R(5D)-substitution); control of the AADH-bands: 1 = *Aadh-A1* (located in 5AL, 2 = *Aadh-D1* (located in 5DL), 3 = *Aadh-B1* (located in 5BL), 4 = *Aadh-R1* (located in 5RL)

Fig. 2. Proof of the presence of the wheat chromosome arm 5DL in some dh-lines (lanes 2–5) from IJ 98 = Amadeus 7143 × dh 5841 by NADP-dependent aromatic alcohol dehydrogenases

Skdh-D1/Skdh-R1, tested as marker for 5DS/5RS), forms two different gametes of the same frequency after meiosis. Consequently, anther culture regenerants with 5DS (*Skdh-D1*) and 5RS (*Skdh-R1*) have been awaited at the ratio 1:1 and therefore, our result fits the theoretical expectations.

The findings show that the use of anther culture does not necessarily result in gametic selection as it is observed for the 1BL.1RS translocation (Henry *et al.*, 1993). In analogy to the 1AL.1RS chromosome, transmitted without distortions of segregation (Müller *et al.*, 1996), the reslults show no shifts of frequency of pollen-derived dh-lines for 5DL.5RS.

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EXCISION OF MAIZE *Ac/Ds* TRANSPOSABLE ELEMENTS AND ITS FREQUENCY IN TRANSGENIC WHEAT REVEALED BY EXPRESSION OF MARKER GENES

EXCIZE TRANSPOZONŮ *Ac/Ds* KUKUŘICE A JEJICH ČETNOST V TRANSGENNÍ PŠENICI ZJIŠŤOVANÁ NA ZÁKLADĚ EXPRESE MARKEROVANÝCH GENŮ

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ABSTRACT: In wheat, no successful transposon tagging has been reported. We have previously produced and established three fixed wheat lines which were constitutively expressing the maize *Activator* (*Ac*) transposase gene. To investigate the activation and excision of the maize transposable elements in the transgenic wheat plants, plasmid DNAs containing the *Dissociation* (*Ds*) element located between a promoter sequence and a reporter gene for β-glucuronidase (*Gus*) or hygromycin resistance (*hph*) were biolistically introduced into scutellar tissues of the *Ac* lines. Excision of *Ds* resulted in the restoration of the *Gus* or *hph* expression and in the generation of GUS-positive or hygromycin-resistant regenerants. The *Ds*-excision frequency in the transgenic wheat, estimated based on the relative GUS activity, was similar to that in transgenic rice. *Ds* lines possessing the interrupted *hph* gene were also produced, and scutellar tissues of F_2 embryos from crosses between the *Ac* and *Ds* lines were cultured on the solid medium containing hygromycin. Several hygromycin-resistant plants were recovered after *trans*-activation and excision of the *Ds* element from a marker gene in the F_2s. These results suggest a possibility of developing a two-element system for transposon tagging in wheat.

Keywords: *Ac/Ds* transposable elements; particle bombardment; phenotypic assay; *Triticum aestivum* L.; two-element system

ABSTRAKT: U pšenice není popsáno úspěšné značkování transpozonů. Již dříve jsme vytvořili tři stálé linie pšenice konstitutivně exprimující kukuřičný gen *Activator* (*Ac*) transposasy. Pro studium aktivace a excize kukuřičných transpozonů v transgenních rostlinnách pšenice byla biolisticky (bombardováním částicemi) do scutelárního pletiva *Ac*-linií vnesena plasmidová DNA obsahující *disociační* (*Ds*) element lokalizovaný mezi sekvencí promotoru a reporterovým genem pro rezistenci k β-glucuronidase (GUS) nebo hygromycinu (*hph*). Excize *Ds* měla za výsledek obnovení exprese *Gus* nebo *hph* a tvorbu GUS-pozitivních nebo hygromycin-rezistentních regenerantů. Četnost *Ds*-excize v transgenní pšenici, odhadnutá na základě relativní aktivity GUS, byla podobná jako u transgenní rýže. Byly rovněž vytvořeny *Ds*-linie obsahující přerušený gen *hph*. Skutelární pletivo embryí F_2 z křížení linií *Ac* a *Ds* bylo pěstováno na pevném médiu obsahujícím hygromycin. Po transaktivaci a excizi *Ds*-elementů markerového genu bylo v generacích F_2 nalezeno několik rostlin odolných k hygromycinu. Tyto výsledky poukazují na možnost vytvoření dvousložkového systému pro značkování transpozonů v pšenici.

Klíčová slova: transpozony *Ac/Ds*; bombardování částicemi; fenotypický test; *Triticum aestivum* L.; dvousložkový systém

INTRODUCTION

Transposon mutagenesis has become a powerful tool for the isolation of agronomically important genes in maize, *Arabidopsis* and other plant species. The maize *Activator* (*Ac*) and *Dissociation* (*Ds*) elements are the best studied and characterized transposable elements in plants (reviewed by Fedoroff, 1989). In wheat, however, no successful transposon tagging has been reported and the information for the heterologous maize *Ac/Ds* transposition is very limited.

The phenotypic assay developed by Baker *et al.* (1987) has been useful for analyzing the transposition by the restored expression of a marker gene after the excision of a transposable element in the heterologous host plants. In both monocotyledonous and dicotyledonous plants,

excision and transposition of the *Ac/Ds* transposable elements have been observed and the sequence alterations at the excision sites have been demonstrated (Fedoroff, 1989). Recently, a simple transient assay for the *Ac/Ds* activity by particle bombardment has been developed in cells of intact barley tissues based on the GUS assay (McElroy *et al.*, 1997). This biolistic assay was proven to be effective to estimate the transposase activity in wheat cells (Takumi *et al.*, 1999a, b). However, information on the developmental regulation of the *Ds* excision has also been very limited in wheat. In our previous study, we established transgenic wheat lines that were expressing the maize *Ac* transposase gene, i.e., the transposase gene was precisely processed and an active transposase protein was synthesized (Takumi *et al.*, 1999a). To examine the frequency and pattern of the *Ds* excision, we introduced the maize *Ds* transposable element located between promoter sequence and the *Gus* reporter gene into the transgenic wheat line expressing the *Ac* transposase gene.

Trans-activation of the *Ds* element in immature embryos

To examine the *Ds* excision by the phenotypic assay (Baker *et al.*, 1987), we introduced either pSP-WDV-Act1.GUS.N alone or a combination of pCKR532 and pSP-WDV-Act1(DsBar)GUS.N into immature embryos, and observed the *Gus* expression. pSP-WDV-Act1(DsBar)-GUS.N and pSP-WDV-Act1.GUS.N respectively contains the rice actin 1 gene (*Act1*) promoter and *Gus* gene with and without the *Ds* element that was originally derived from waxy-*m9* allele (McElroy *et al.*, 1997). In pSP-WDV-Act1(DsBar)GUS.N, the expression of the *Gus* gene can be induced only after the *Ds* element is excised (Fig. 1). The modified *Ds* element has the bialaphos-resistant marker gene (*bar*) for selection of transformants. pCKR532 contains the *Ac* transposase coding region under control of the 35S promoter (Shimamoto *et al.*, 1993). This *Ac* transposase gene, which was originally derived from the entire *Ac* element of the maize waxy-*m7* allele (Klösgen *et al.*, 1986), lacks the inverted repeats of the *Ac* element and thus cannot transpose by itself.

Immature embryos of a Japanese common wheat (*Triticum aestivum* L.) cv. Akadaruma, an emmer wheat accession *T. durum* var. *agricunum*, and two barley (*Hordeum vulgare* L.) cvs. Ruen and Lenins were dissected 14-days after anthesis. The immature caryopses from greenhouse-grown plants were sterilized in 70% ethanol and the dissected immature embryos were placed with scutellar tissues exposed on Linsmaier-Skoog (LS) medium (Linsmaier and Skoog, 1965) containing 2 mg/l of 2,4-D and 0.25% (w/v) Gelrite (Merck). Seven-day cultured embryos were bombarded with pSP-WDV-Act1.GUS.N or with pCKR532 and pSP-WDV-Act1(DsBar)GUS.N. The conditions of particle bombardment and purification of the bombarded plasmids were as described previously (Takumi *et al.*, 1994). The bombarded embryos were incubated at 26°C for two days prior to the assay for GUS

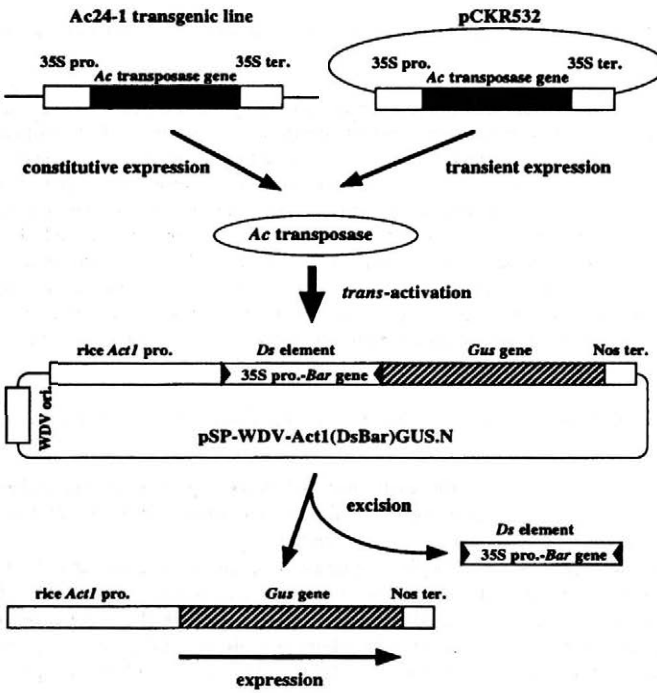

Fig. 1. Phenotypic assay of the maize *Ac/Ds* elements using the GUS activity in this study. *Ac* transposase protein derived from a genomic *Ac* region of the Ac24-1 line and plasmid DNA of pCKR532 can activate the DsBar cassette placed between the rice *Act1* promoter and the *Gus* gene. The *Ds* excision restores the expression of the *Gus* gene. The *Gus* expression can be detected as a blue spots in the GUS assay

Table 1. Excision frequencies of the *Ds* transposable element from the bombarded plasmid containing the interrupted *Gus* gene in cultured immature embryos of wheat and barley

Accessions	No. blue spots (mean±SE) per embryo bombarded with pSP-WDV-Act1.GUS.N	No. blue spots (mean ± SE) per embryo bombarded with pSP-WDV-Act1(DsBar)- GUS.N and pCKR532	Excision frequency (%)
Akadaruma	43.27 ± 1.94	1.07 ± 0.25	2.47
agricunum	32.65 ± 6.39	1.28 ± 0.35	3.91
Lenins	7.29 ± 1.20	0.54 ± 0.22	7.39
Ruen	10.31 ± 2.10	1.00 ± 0.22	9.70

activity. GUS activity was histochemically assessed (Takumi *et al.*, 1994) and the average number of blue spots per embryo was determined. In the case with pSP-WDV-Act1.GUS.N, 7 to 43 blue-staining cells (blue spots) per embryo were recognized in the four accessions of wheat and barley (Table 1). No blue spots were observed in the cultured embryos bombarded only with pSP-WDV-Act1(DsBar)GUS.N (data not shown). A few blue spots were detected in the cultured embryos of all the four accessions after bombardment with pSP-WDV-Act1-(DsBar) GUS.N and pCKR532. The number of blue spots per embryo was much less than that bombarded with pSP-WDV-Act1.GUS.N. The results demonstrated that the excision of *Ds* elements occurred only in the presence of the *Ac* transposase gene. The *Ds*-excision frequency was calculated based on the average number of blue spots per embryo bombarded with pSP-WDV-Act1(DsBar)-GUS.N relative to that with pSP-WDV-Act1.GUS.N. The excision frequency was higher in the barley cultivars than that in the wheat accessions, although the number of blue spots was lower in barley.

Transient *Gus* expression after *Ds* excision in transgenic wheat plants

Immature embryos of an *Ac*-expressing transgenic line, Ac24-1, were used to detect the *Ds* excision. Ac24-1 was derived from a common wheat cultivar Norin 12 and it constitutively expressed the transposase gene of the *Ac* element (Takumi *et al.*, 1999a). However, the *bar* gene, which was used as a selection marker, was not expressed in Ac24-1, most likely due to transcriptional silencing. Immature embryos were dissected and placed with scutellar tissues exposed on LS medium containing 2 mg/l of 2,4-D. The immature embryos bombarded with either pSP-WDV-Act1(DsBar)GUS.N or pSP-WDV-Act1.GUS.N were incubated at 26°C for two days prior to the assay for GUS activity.

Blue spots could be recognized only after the *Ds* excision in the Ac24-1 line. The number of blue spots after bombardment with pSP-WDV-Act1(DsBar)GUS.N was much less than that with pSP-WDV-Act1.GUS.N. The result indicated that the *Ac* transposase gene was precisely transcribed and functionally active transposase was produced in the transgenic wheat plants. The relative activity of the *Gus* gene was determined using GUS activity value (number of blue spots) of cultured embryos bombarded with pSP-WDV-Act1.GUS.N. as reference. The excision frequency of the *Ds* element was estimated based on the relative activity of the *Gus* gene. In this study, the excision frequency estimated in the Ac24-1 line was 2.39%. This frequency was higher than our previous data obtained using the same line (Takumi *et al.*, 1999a). Table 2 summarizes the *Ds*-excision frequencies from pSP-WDV-Act1(DsBar)GUS.N in the transgenic wheat and rice cells. In wheat, the *Ds*-excision frequency varied from 0.17% to 2.39%, the range of which was similar to that in rice.

Table 2. Excision frequencies of the *Ds* element in *Ac*-expressing cells of transgenic wheat and rice

Plant materials	Lines	Excision frequencies
WHEAT		
Transgenic plant	Ac24-1	0.0239*
	Ac24-1	0.0098
	Ac3-1	0.0056
Transgenic callus	H234,532-4	0.0017
	H234,532-6	0.0023
	H262-2	0.0024
	H234,532-3	0.0046
	H234,532-5	0.0047
	H234,532-8	0.0059
	H234,532-7	0.0079
	H262-1	0.0122
		0.0017–0.0239
RICE		
Transgenic *indica* callus	AHY41	0.022
	AHY45	0.007
	AHY48	0.005
	BA15-1	0.003
	BA15-2	0.005
	BA15-3	0.003
Transgenic *japonica* callus	AHY5	0.003
	AHY10	0.016
		0.003–0.022

*Data were obtained in this study. All others in transgenic plants and callus of WHEAT were respectively from Takumi *et al.* (1999a, b) and those in RICE were from Solis *et al.* (1999)

Dosage effect on the *Ac* activity is determined by the *Ac* copy number and the level of *Ac* transcript in the cells. The positive dosage effect of *Ac* and *Ds* transposition has been observed in maize kernels (Heinlein, 1996), tobacco cotyledons (Scofield *et al.*, 1993) and Arabidopsis (Swinburne *et al.*, 1992) at low levels of *Ac* transcripts. However, at higher leveles of *Ac* expression, *Ac* had negative dosage effect on transposition in tobacco (Scofield *et al.*, 1993) and maize (Heinlein, 1996). The *Ds*-excision frequency varied significantly (at the 1% level) among the transgenic callus lines (Takumi *et al.*, 1999b). In these lines, the negative dosage effect was observed. A high level of *Ac* transcript also did not lead to a high frequency of the *Ds* excision in transgenic rice cells (Solis *et al.*, 1999). It is however unknown if dosage effect of the *Ac* element occurred in the transgenic wheat plants, as were observed in tobacco and maize plants.

Stable transformants generated after the *Ds* excision

Transgenic wheat plants were produced according to the method established previously (Takumi and Shimada, 1996). Seven to 10-day-old immature embryos of Ac24-1 were bombarded with pSP-WDV-Act1(DsBar)-GUS.N or pCKR234 and transferred to selection medium after one day. pCKR234 contains the *Ds* element, which lacks the 1.6 kb *Hin*dIII fragment of *Ac* element, inserted between the 35S promoter and the *hph* gene (Shimamoto *et al.*, 1993). The selection medium comprised of LS medium with 2 mg/l of 2,4-D and 5 mg/l of bialaphos or 50 mg/l of hygromycin B. After about one month, bialaphos-resistant tissues were transferred to 2,4-D-free LS medium containing 4 mg/l of bialaphos or 40 mg/l of hygromycin B for regeneration.

GUS activity was demonstrated in the bialaphos-resistant callus and the regenerated plants were bombarded with pSP-WDV-Act1(DsBar)GUS.N (Fig. 2). In the bialaphos-resistant callus, the *Gus* expression was observed as a blue region. These callus derived from the bombarded embryos grew to form bialaphos-resistant sectors, in which the *Ds* elements were excised and the *Gus* genes were integrated into the host genome. In regenerated plants independently derived from some bialaphos-resistant callus, all tissues were stained blue, indicating the constitutive expression of the *Gus* gene. This result suggested that the *Ds* element was excised during the early stages of regeneration when the pSP-WDV-Act1(DsBar)-GUS.N was introduced into the cells. In contrast, other regenerated plants showed chimeric patterns with and without the GUS staining. The GUS-staining regions differed among the chimeric plants in both leaves and roots. It was unclear whether the regenerated plant was originally derived from one cell. The chimeric patterns, however, indicated the time of excision of the *Ds* element during the regeneration from bialaphos-resistant callus.

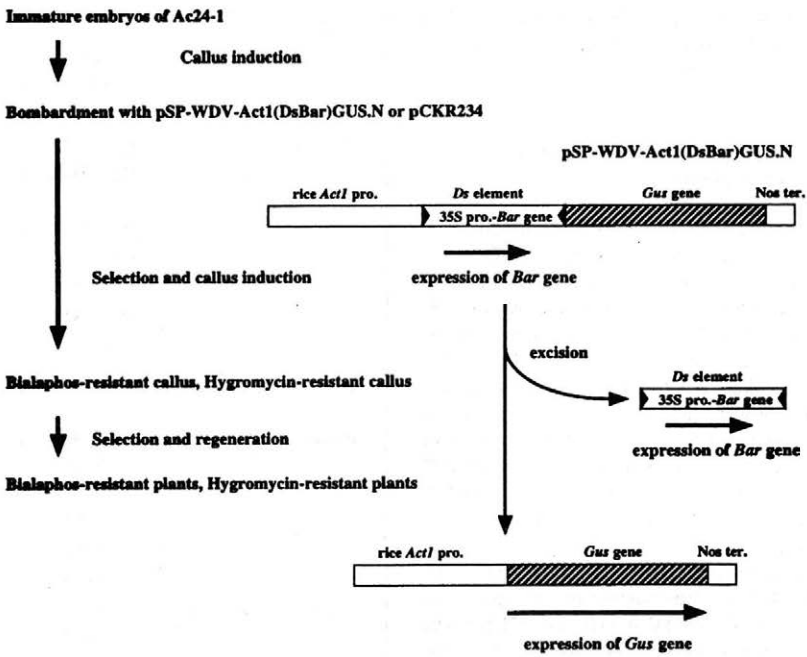

Fig. 2. A flow chart of the experiment to produce stable transformants generated after the *Ds* excision. After bombardment with pSP-WDV-Act1(DsBar)GUS.N, immature embryos of the Ac24-1 line were transferred to the selection medium containing bialaphos. The GUS activity was assessed in the bialaphos-resistant callus and in the regenerated plants. After *Ds*-excision from pCKR234, hygromycin-resistant plants were recovered on the selection medium containing hygromycin

The *Ds* excision occurred at the early stage of the plant regeneration.

More than 1,500 immature embryos of Ac24-1 were bombarded with pCKR234. Forty-one hygromycin-resistant plants were independently generated on the selection medium. Southern blot analysis confirmed the re-integration of the excised *Ds* element into wheat genome. In some regenerated plants, additional bands were observed in the Southern blot pattern probed with 1.8 kb region of the *Ds* element (data not shown). This result suggested that the *Ds* element was first excised from the plasmid sequence and re-integrated into the other region of the genome.

Activation of the *Ds* element in crosses between *Ac* and *Ds* lines

We examined the recovery of the hygromycin-resistant plants after trans-activation and excision of the *Ds* element from a marker gene in crosses between the *Ac* and *Ds* lines. *Ds* lines containing the interrupted *hph* gene were also produced. Scutellar tissues of F_2 embryos were cultured on the LS medium containing 2 mg/l of 2,4-D and 50 mg/l of hygromycin B for 2 weeks, and transferred to the 2,4-D-free medium containing 40 mg/l of the hygromycin. More than 1,300 F_2 embryos derived from seven independent F_1 plants were cultured, and three hygromycin-resistant plants were recovered. Our result suggests the possibility to develop a two-element system for transposon tagging in wheat.

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PEA (*PISUM SATIVUM* L.) MENDELIAN GENES CONTROLLING DEVELOPMENT OF NITROGEN-FIXING NODULES AND ARBUSCULAR MYCORRHIZA*

MENDELOVSKÉ GENY HRACHU (*PISUM SATIVUM* L.) KONTROLUJÍCÍ VÝVOJ NODULÍ FIXUJÍCÍCH DUSÍK A ARBUSKULÁRNÍ MYKORHIZU

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ABSTRACT: Pea is one of the most developed model plants for genetic studies of developmental processes leading to the formation of nitrogen-fixing symbiosis (NFS) and arbuscular mycorrhiza (AM). More than 200 symbiotic mutants with respect to NFS were independently obtained in different laboratories throughout the world. More than 100 mutants have been involved in genetic analysis by several research teams. As a result, more than 40 symbiotic genes have been identified up to date. Analysis of mutant phenotypes allowed to subdivide the NFS establishment and the AM formation into discrete developmental stages controlled by different groups of plant genes. Those stages can be considered as elementary traits for genetic and phenotypic analysis. At least 8 such stages have been identified for NFS and 3 stages for AM. The existence of common plant genes and plant molecular products for both symbioses makes it possible to conclude, that the legumes have a joint genetic system controlling the development of a tripartite symbiosis.

Keywords: plant–microbe interactions; nitrogen-fixing nodules; arbuscular mycorrhiza; tripartite symbiosis; pea; *Pisum sativum* L.; symbiotic genes

ABSTRAKT: Hrách je jedním z nejrozvinutějších rostlinných modelů pro genetické studie vývojových procesů vedoucích ke vzniku symbiotické fixace dusíku (NFS) and arbuskulární mykorhizy (AM). Přes 200 symbiotických mutantů ve vztahu k NFS bylo nezávisle získáno v různých laboratořích ve světě a více než 100 mutantů bylo zařazeno do genetických analýz. Doposud bylo identifikováno přes 40 genů pro symbiózu. Analýza fenotypů mutantů umožnila rozčlenění procesu vzniku NFS a tvorby AM do diskrétních vývojových stadií kontrolovaných odlišnými skupinami rostlinných genů. Tato stadia mohou být považována za elementární znaky pro genetickou a fenotypickou analýzu. Nejméně osm takových stadií bylo zjištěno u NFS a tři stadia u AM. Existence obecných rostlinných genů a rostlinných molekulárních produktů pro obojí symbiózu dovoluje učinit závěr, že motýlokvěté rostliny mají společný genetický systém kontrolující vývoj třístadiové symbiózy.

Klíčová slova: interakce rostlina–mikroorganismus; noduly fixující dusík; arbuskulární mykorhiza; třístadiová symbióza; hrách; *Pisum sativum* L.; symbiotické geny

INTRODUCTION

The first indications about an active role of the host plant in the establishment of endosymbiotic systems appeared many years ago. Vorhees (1915) demonstrated that some soybean cultivars are not nodulated with certain strains of nodule bacteria. Later in the middle of thirties Razumovskaia (1937) and Govorov (1937) found pea varieties originated from Afghanistan which do not form nodules with the strains of nodule bacteria isolated from European soils whereas other peas do. Much later, by the end of sixties T. A. Lie (1971) began his work on identifi-

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Table 1. Pea (*Pisum sativum* L.) symbiotic mutants obtained in different genotypes

Genotypes (Author(s), Country)	Mutant phenotypes				
	Nod$^-$	Nod$^{+/-}$	Fix$^-$	Nod^{++}	Total
RONDO (E. Jacobsen *et al.*, The Netherlands)	3	8	1	1	13
SPARKLE (T. A. LaRue *et al.*, USA)	11	12	4	0	27
FINALE (K. J. Engvild, Denmark)	22	1	11	1	35
FRISSON (G. Duc, M. Sagan, France)	19	1	19	25	64
RAMONSKY-77 (K. K. Sidorova, L. P. Uzhintseva, Russia)	1	0	1	1	3
SPRINT-2 (A. Y. Borisov *et al.*, Russia)	3	0	1	0	4
SGE (V. E. Tsyganov *et al.*, Russia)	20	3	33	1	57
Total	79	25	70	29	203

Compiled data from G. Duc & M. Sagan, personal communication, Tsyganov *et al.*, unpublished results and data published in literature (Jacobsen, 1984; Jacobsen and Feenstra, 1984; Kneen and LaRue, 1984a, 1986, 1987, 1988; Engvild, 1987; Postma *et al.*, 1988, 1990; Kneen *et al.*, 1989, 1990; Duc and Messager, 1989; Weeden *et al.*, 1990; Borisov *et al.*, 1992, 1994; Sidorova and Uzhintseva, 1992; Sagan *et al.*, 1993; Tsyganov *et al.*, 1994)

cation of pea symbiotic genes based on natural genetic polymorphism and the first symbiotic genes (named *sym*-genes) controlling strain specific nodulation were identified (Lie, 1984). However, great achievements in this field were attained only after application of experimental mutagenesis.

As a result of all efforts throughout the world on the isolation of pea symbiotic mutants with abnormalities of nodule formation and function, more than two hundred independently obtained symbiotic mutant lines are known to date (Table 1). It is generally accepted that newly isolated symbiotic mutants should be classified according to four main phenotypes: Nod$^-$ (no nodules), Nod$^{+/-}$ (few or no nodules), Fix$^-$ (ineffective nodules), Nod^{++} (supernodulation) (Sagan *et al.*, 1994). More than one hundred of isolated symbiotic mutants were then involved in complementation analysis in various laboratories and more than forty pea symbiotic genes were identified so far (Table 2).

The majority of the genetically characterized mutants were phenotypically studied in detail in order to identify nodule developmental stages blocked by mutations in certain identified genes (Postma *et al.*, 1988, 1990; Kneen *et al.*, 1990; Guinel, LaRue 1991; Borisov *et al.*, 1992, 1994; Markwei, LaRue, 1992, 1997; Sagan *et al.*, 1993, 1994; Novak *et al.*, 1995; Tsyganov *et al.*, 1998, 1999; Morzhina *et al.*, 2000). This characterization allowed a subdivision of nodule morphogenesis into eight discrete developmental stages (Table 3). The obtained results made it necessary, to modify the previously used system of phenotypic codes describing the process of symbiotic nodule development (Vincent, 1980; Caetano-Anolles and Gresshoff, 1991), because new nodule developmental stages controlled by plant genes were discovered (Tsyganov *et al.*, 1998). So far the sequence of nodule developmental stages was defined as follows: (i) root hair curling (Hac), (ii) infection thread growth initiation (Iti), (iii) infection thread growth inside root hair (Ith), (iv) infection thread growth inside root tissue (Itr), (v) infection thread growth inside nodule tissue (Itn), (vi) infection droplet differentiation (Idd), (vii) bacteroid differentiation (Bad) and nodule persistence (Nop).

At the end of the eighties the first report about symbiotic mutants with abnormalities of nodule development and blocks of arbuscular mycorrhiza (AM) formation appeared (Duc *et al.*, 1989). Consequently, the analysis of the ability-to form AM was included into the scheme of phenotypic characterization of mutants, obtained in various legumes. With respect to pea at least three AM developmental stages (Table 3), controlled by at least eight plant genes (of those involved in nodule formation), were revealed to date. These stages are (i) infecting hyphae growth from appressorium (Myc^1), (ii) arbuscule formation (Myc^2) (Gianinazzi-Pearson *et al.*, 1991, Gianinazzi-Pearson, 1996) and (iii) intensity of host plant root colonization (Myc$^{+/-}$) (authors's data).

All the data presented above allowed us to conclude that at present pea is one of the most genetically developed plants for studying processes leading to the formation of two endosymbioses: nitrogen-fixing nodules and AM. The existence of common plant genes and plant molecular products for both symbioses makes it possible to conclude that the legumes have in common a genetic system controlling the development of a tripartite symbiosis. It can also be postulated, that the plant genetic system for NFS was based in its evolution on that for AM (Gianinazzi-Pearson, 1997). The latter facts are very important for breeding process to create new commercial legume cultivars with high symbiotic potential.

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Table 2. Pea (*Pisum sativum* L.) symbiotic genes identified in the course of studying root nodule formation

Gene symbols	Phenotypes	Mutant lines	References
Sym1 = sym2	Nod$^{+/-}$	JI 1357 (registered type line)	Lie (1971); Holl (1975); Kneen, LaRue (1984b); Kozik *et al.* (1995)
sym3	Fix$^-$	JI 1357 (registered type line)	Holl (1975)
Sym4	Nod$^-$	JI 261	Lie (1984)
sym5	Nod$^-$	E2, R88, E77, E111, E143, E166, E169	Kneen, LaRue (1984a)
sym6	Fix$^-$	JI 1357 (registered type line)	Lie, Timmermans (1983); Lie *et al.* (1987)
sym7	Nod$^-$	E69, N12, RisNod14	Engvild (1987); Kneen *et al.* (1994); Duc, Sagan, p.c.
sym8 = sym20	Nod$^-$	E14, R19, R25, R80, RisNod10, RisNod13, RisNod19, RisNod21, RisNod25, Sprint-2Nod$^-$-1, Sprint-2Nod$^-$-2	Engvild (1987); Borisov *et al.* (1994); Kneen *et al.* (1994); Duc, Sagan, p.c.
sym9	Nod$^-$	R72, P54	Duc, Messager (1989); Kneen *et al.* (1994); Duc, Sagan, p.c.
sym10	Nod$^-$	P5, P7, P8, P9, P10, P56, RisFixG	Engvild (1987); Duc, Messager (1989); Duc, Sagan, p.c.
sym11	Nod$^-$	N24	Kneen *et al.* (1994)
sym12	Nod$^{+/-}$	K5	Postma *et al.* (1988)
sym13	Fix$^-$	E135f, E136, P58	Kneen *et al.* 1990; Sagan *et al.* (1993)
sym14	Nod$^-$	E135n, SGENod$^-$-2	Kneen *et al.* (1990); Tsyganov *et al.* (1999)
sym15	Fix$^-$	E151	Kneen *et al.* (1994)
sym16	Fix$^-$	R50	Kneen *et al.* (1994)
sym17	Nod$^{+/-}$	R82	Kneen *et al.* (1994)
sym18	Nod$^{+/-}$	E54	LaRue *et al.* (1996)
sym19	Nod$^-$	P4, P6, P55, NEU5, NMU1, RisNod2, RisNod7, RisNod16, RisNod20	Kneen, LaRue (1988); Duc, Messager (1989); Weeden *et al.* (1990); Duc, Sagan, p.c.
sym21	Nod$^{+/-}$	E132	Markwei, LaRue (1997)
Sym22	Nod$^{+/-}$	JI 1794	LaRue, Weeden (1992)
sym23	Fix$^-$	P59	Duc, Messager (1989); Duc, Sagan, p.c.
sym24	Fix$^-$	P60	Duc, Messager (1989); Duc, Sagan, p.c.
sym25	Fix$^-$	P14, P17, P19, P61	Duc, Messager (1989); Duc, Sagan, p.c.
sym26	Fix$^-$	P63, RisFixM, RisFixT	Engvild (1987); Duc, Messager (1989); Duc, Sagan, p.c.
sym27	Fix$^-$	P12, RisFixO	Engvild (1987); Duc, Messager (1989); Duc, Sagan, p.c.
sym28	Nod^{++}	P64, P77	Sagan, Duc (1996)
sym29	Nod^{++}	P87, P88, P89, P90, P91, P93, P94	Sagan, Duc (1996)
sym30	Nod$^-$	P1, P2, P3, P53, RisNod6, RisNod9, RisNod22	Engvild (1987); Duc, Messager (1989); Duc, Sagan, p.c.
sym31	Fix$^-$	Sprint-2Fix$^-$	Borisov *et al.* (1992, 1997)
sym32	Fix$^-$	RisFixL, RisFixO	Engvild (1987); Duc, Sagan, p.c.
sym33	Fix$^-$	RisFixU, SGEFix$^-$-2	Engvild (1987); Tsyganov *et al.* (1998); Duc, Sagan, p.c.
sym34	Nod$^-$	RisNod1, RisNod23, RisNod30	Engvild (1987); Duc, Sagan, p.c.
sym35	Nod$^-$	RisNod8, SGENod$^-$-1, SGENod$^-$-3	Engvild (1987); Tsyganov *et al.* (1999); Duc, Sagan, p.c.
sym36	Nod$^-$	RisNod24, RisNod26	Engvild (1987); Duc, Sagan, p.c.
sym37	Nod$^{+/-}$	RisNod4	Engvild (1987); Duc, Sagan, p.c.
sym38	Nod$^-$	RisFixF, SGENod$^-$-4, SGENod$^-$-8	Engvild (1987); Tsyganov *et al.* (1994, unpublished results); Duc, Sagan, p.c.
sym39	Nod$^{+/-}$	P57	Sagan *et al.* (1994); Duc, Sagan, p.c.
sym40	Fix$^-$	SGEFix$^-$-1	Tsyganov *et al.* (1998)
sym41	Fix$^-$	RisFixA	Engvild (1987); Tsyganov *et al.* (unpublished results)
nod1, nod2	Nod^{++}	Parvus	Gelin, Blixt (1964)
nod3	Nod^{++}	nod3, P79, RisFixC	Jacobsen, Feenstra, (1984); Engvild (1987); Duc, Sagan, p.c.
Total – 41	**4**	**108**	

*p.c. – personal communication

Table 3. Pea (*Pisum sativum* L.) genes controlling developmental stages of root nodules and AM. Regular font marks genes implicated in nodule formation only, bold font marks genes implicated in the development of both symbiotic systems (For details see text)

Hac	Iti	Ith	Itr	Itn	Idd	Bad	Nop
sym8	*sym7*	*sym2*	*sym5*	***sym33***	***sym40***	*sym31*	*sym13*
sym9	***sym14***	***sym36***	*sym34*		***sym41***	*sym32*	*sym25*
sym10	*sym35*						*sym26*
sym19							*sym27*
sym30							
Myc^{-1}	**Myc$^{+/-}$**	**Myc^{-2}**		**Myc$^{+/-}$**			

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GENETIC ANALYSIS OF SEED COLOUR IN RAPESEED (*BRASSICA NAPUS* L.)

GENETICKÁ ANALÝZA BARVY SEMEN ŘEPKY (*BRASSICA NAPUS* L.)

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ABSTRACT: The combination of genetic and environmental factors gives oilseed rape (*Brassica napus*) a range of seed colours. In the course of a breeding programme F_1 material was derived from crosses between dark-seeded high-erucic acid rapeseed lines and a true-breeding yellow-seeded *B. napus* (00). Both, inbred and doubled-haploid (DH) lines were generated. The genetic segregation of the DH populations fitted a 1:6:1 ratio; hence seed colour has shown to be inherited in an additive manner: Black seeds occur when all three loci are homozygously dominant, true yellow seediness is only manifested in the case of three homozygous recessive alleles, whereas all other genetic constitutions result in a more or less brown seed colour. In addition to visual assessment of seed colour major seed traits were analytically determined and screened by using both, a digital optical-picture analysis system and non-destructive near-infrared reflectance spectroscopy (NIRS).

Keywords: *Brassica napus*; oilseed rape; *Arabidopsis thaliana*; yellow seed colour; oil; protein; doubled-haploid lines; inheritance

ABSTRAKT: Kombinací genetických faktorů a vlivů prostředí vzniká škála barev semene řepky olejné (*Brassica napus*). V průběhu šlechtitelského programu byl získán materiál F_1 z křížení tmavosemenných linií s vysokým obsahem kyseliny erukové a neštěpící žlutosemenné *B. napus* (00). Z potomstva byly získány jak inbrední, tak i dihaploidní (DH) linie. Štěpení populace DH odpovídalo poměru 1 : 6 : 1, takže bylo zřejmé, že barva semene se dědila aditivně: černá semena se objevovala, pokud všechny tři lokusy byly homozygotně dominantní, žlutá semena pouze za přítomnosti tří homozygotních recesivních alel, zatímco všechny ostatní genetické konstituce se projevovaly více nebo méně hnědým zbarvením semen. Mimo vizuální hodnocení barvy semen byly hlavní znaky semen hodnoceny současně digitální analýzou optického obrazu a nedestruktivní infračervenou spektroskopií (NIRS).

Klíčová slova: *Brassica napus*; řepka olejná; *Arabidopsis thaliana*; žlutá barva semene; olej, protein; dihaploidní linie; dědičnost

INTRODUCTION

Following oil extraction the meal of oilseed rape (*Brassica napus*) contains about 40% of protein with a well balanced amino acid composition. Among other anti-nutritional compounds, including glucosinolates, phytate, sinapine, phenolic acid and tannins, crude fibre (and substances enclosed by them) affects adversely the usability of rapeseed meal in feed and nutrition. Regarding further improvement of rapeseed, the yellow seed colour is considered to have a pronounced influence on seed quality leading to a concomitant increase of digestible energy and protein content in the meal (Bell, 1993; Slominski, 1997; Slominski *et al.*, 1999; cf. Friedt and Lühs, 1999). This is explained through the characteristically thinner seed coat of yellow as compared to black seeds (Stringam *et al.*, 1974; Theander *et al.*, 1977; Shirzadegan and Röbbelen, 1985; Bechyně 1987; Morgan *et al.*, 1998). Light seed colour and low fibre content are considered to coincide because the biochemical pathways leading to lignin and seed testa pigment biosynthesis have common precursors (e.g., 4-coumarate) along the phenylpropanoid pathway. Phenylpropanoids include a wide range of plant phenolic compounds, such as flavonoids, stilbenes, cumarins and tannins, as well as cell wall constituents, like lignin, suberin or cutin. Flavonoids serve a variety of functions in higher plants. These compounds are present at high levels in most seeds and they appear to play vital roles in defence against pathogens or pests and contribute to physiological functions, such as seed maturation, dormancy, viability and seedling vigour as well as protection against ultraviolet (UV) light. At the same time, particular subclasses of flavonoids, such as the proanthocyanidins (condensed tannins),

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negatively impact the use of seeds in animal feed and can add undesirable qualities to food products for human consumption (Dixon *et al.*, 1996; Graham, 1998; Shirley, 1996, 1998; Weisshaar and Jenkins, 1998; Whetten *et al.*, 1998; Debeaujon *et al.*, 2000).

In general, the introgression of genes encoding seed pigmentation (e.g., from related *Brassica* species) and subsequent expression of seed colour in *B. napus* can be complex due to allotetraploidy ($2n = 4x = 38$), multiple gene control, predominantly maternal determination and environmental impact (Van Deynze *et al.*, 1993; Rashid *et al.*, 1994; Tang *et al.*, 1997; Meng *et al.*, 1998). For *A. thaliana* different mutants have been identified or in a few cases genes have already been cloned showing the *transparent testa* (*tt*) phenotype which is associated with yellow or ochre seed colour due to lack of anthocyanin (Koornneef, 1981; Shirley *et al.*, 1995; Wisman *et al.*, 1998; Focks *et al.*, 1999; Walker *et al.*, 1999). Particularly, interest exists to investigate if any of the known *Arabidopsis tt*-mutants correspond to one or more of the *B. napus* loci encoding yellow seediness or if novel sources for this agronomically important trait could be exploited for quality breeding in rapeseed. However, the biochemical lesions leading to the lack of pigmentation in *B. napus* have not been identified, yet. Comparative mapping experiments have revealed extensive genome colinearity between *Brassica* species and *A. thaliana* due to the high sequence conservation. On average, gene sequences show 87% sequence identity (Lydiate *et al.*, 1993; Lagercrantz and Lydiate, 1996; Lagercrantz, 1998; Cavell *et al.*, 1998). Genome sequencing projects are providing novel approaches for identifying plant biosynthetic genes of nutritional importance. Recently, the term "nutritional genomics" has been used in order to describe work at the interface of plant biochemistry, genomics, and nutrition (cf. Schmidt, 1998; DellaPenna, 1999). Therefore, exploiting the knowledge about *Arabidopsis* genomics in improving the nutritional value of rapeseed through the development of yellow-seeded *B. napus* varieties would have a strong impact on the production of oilseed rape (Friedt and Lühs, 1999).

MATERIAL AND METHODS

In the course of a quality breeding programme yellow-seeded *B. napus* lines for better meal quality are developed at our institute. For that purpose, a set of widely divergent *Brassica* materials including *B. napus* were evaluated as sources for yellow seediness. Intensity and stability of the trait 'yellow seed colour' were examined. Yellow-seed pure lines as candidates for the genetic characterisation were developed by microspore culture. At present, a sufficient number of DH lines and corresponding inbred lines is available derived from the F_1 generation of crosses between different dark-seeded *B. napus* lines and a yellow-seeded *B. napus* winter genotype T-25629 as described earlier (Shirzadegan, 1986; Baetzel *et al.*, 1999). The assessment of seed colour was carried out by a digital optical-picture analysis system. Crude seed composition was determined by near-infrared reflectance spectroscopy (NIRS) as described by Van Deynze and Pauls (1994a) and Velasco *et al.* (1996). Chi-square goodness-of-fit tests were used to compare the observed distribution in the DH populations to those predicted by various genetic models for seed colour inheritance (Shirzadegan, 1986; Henderson and Pauls, 1992; Van Deynze and Pauls, 1994b). The data for seed colour were pooled, tested for heterogeneity and fit to appropriate genetic models (cf. Mudra, 1958).

RESULTS AND DISCUSSION

Seed quality assessment

Due to the introgression of the yellow seed trait, an increased variation in seed characters has been generated. Following a phase of seed multiplication in the field a large number of both DH lines and inbred lines (F_4S_3) were developed, respectively. The box plots in Fig. 1 display the variability found in DH lines and corresponding inbred lines (F_4S_3) of two crosses (Gi 3, Gi 4) in comparison to high-yielding varieties used as controls. Due to an increase of protein content the novel rapeseed material – segregating for seed colour – possesses a higher sum of both protein and oil with an average ranging from 71.5 to 72.8% (dry matter based, measured by NIRS).

Among the checks only the high-erucic acid cultivar Maplus (oil + protein = 71.3%) is comparable due to its high oil content (50.6%). Therefore, searching for lines that deviate from the general negative correlation between protein and oil content of the seed (Grami and Stefansson, 1977; Uppström, 1995) by having a high level of both components should help to improve the yield of useful storage products in rapeseed still further. In particular, some of the brown-seeded inbred lines meet this goal. These lines are of primary interest for subsequent breeding programmes.

Inheritance of seed colour

With regard to genetic studies, the DH lines showing a similar variation of seed colour were used as basis for phenotypic classification by a digital optical-picture system (Table 1). Depending on the source of yellow seediness, in most cases a trigenic control of seed colour has been proposed. Due to Shirzadegan (1986) as well as Henderson and Pauls (1992) black or brown seeds are genetically determined by epistatic interactions of dominant alleles according to a black:brown:yellow = 4:3:1 segregation. A similar pattern of genetic control (2:5:1) has been proposed elsewhere (Van Deynze and Pauls, 1994b; Van Deynze *et al.*, 1995). In the present study, the segregation of the DH populations fitted a 1:6:1 ratio

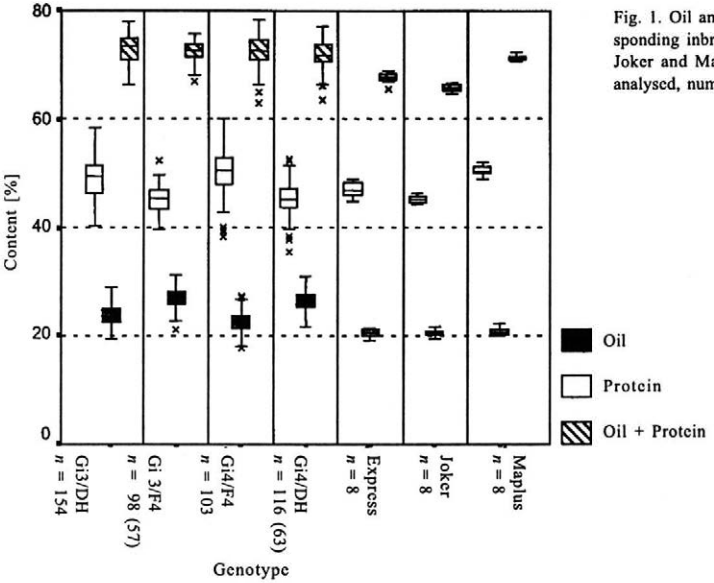

Fig. 1. Oil and protein (% of DM) of DH lines, corresponding inbred populations and the cultivars Express, Joker and Maplus as checks (n – number of samples analysed, number of different DH lines in brackets)

(Table 1), i.e., seed colour appears to be inherited in an additive manner: Black seeds occur when all three major gene loci involved are homozygously dominant, pure yellow seediness is manifested only in the case of three homozygously recessive alleles, whereas all other genetic constitutions result in a more or less brown seed colour. The heterogeneity was tested according to Mudra (1958) indicating that pooled data for all four crosses and the data for each single cross fit the predicted segregation very well. This result provides evidence that the DH populations developed in this study do not exhibit inheritance with epistatic gene action, as assumed by Shirzadegan (1986) for inbred populations.

Prospects for marker-assisted selection

The development of molecular markers linked to gene loci controlling seed colour in *B. napus* has gained importance because selection for yellow seediness is hindered due to pronounced environmental effects, e.g., temperature during seed ripening (Teutonico and Osborn, 1994; Van Deynze *et al.*, 1993, 1995; Upadhyay *et al.*, 1996; Chen *et al.*, 1997; Somers *et al.*, 1999). Based on the DH population segregating for seed colour, fatty acid composition, glucosinolate content and a range of agronomic characters the construction of a skeleton genetic map to localise relevant genes were recently start-

Table 1. Observed and expected segregation for seed colour in selected DH populations (expected frequencies in brackets)

Seed colour*	Genotype	Predicted ratio	Gi 3	Gi 4	Gi 5	Gi 9	Pooled	Total	Heterogeneity
Black ≤ 69	$Bl_1\,Bl_1\,Bl_2\,Bl_2\,Bl_3\,Bl_3$	1	15 (12)	9 (11.3)	8 (7)	6 (5.3)	38 (35.6)		
Brown 70–139	$bl_1\,bl_1\,Bl_2\,Bl_2\,Bl_3\,Bl_3$ $bl_1\,bl_1\,Bl_2\,Bl_2\,bl_3\,bl_3$ $bl_1\,bl_1\,bl_2\,bl_2\,Bl_3\,Bl_3$ $Bl_1\,Bl_1\,Bl_2\,Bl_2\,bl_3\,bl_3$ $Bl_1\,Bl_1\,bl_2\,bl_2\,Bl_3\,Bl_3$ $Bl_1\,Bl_1\,bl_2\,bl_2\,bl_3\,bl_3$	6	71 (72)	74 (67.5)	40 (42)	30 (32.3)	215 (213.8)		
Yellow ≥140	$bl_1\,bl_1\,bl_2\,bl_2\,bl_3\,bl_3$	1	10 (12)	7 (11.3)	8 (7)	7 (5.3)	32 (35.6)		
Total			96	90	56	43	285		
χ^2			1.09	2.68	0.38	0.72	0.54	4.87	4.33
DF			2	2	2	2	2	8	6
P%			50–90	20–50	50–90	50–90	50–90	50–90	50–90

*seed colour assessed by digital optical-picture system; reference dimensions = brightness values

ed. Furthermore, the DH and inbred lines produced in the course of this programme provide a suitable basic material for the establishment and validation of a NIRS rapid screening method determining seed colour and crude fibre content.

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GENETICS OF ERUCIC ACID CONTENT IN *BRASSICA OLERACEA* SEED OIL*

GENETIKA OBSAHU KYSELINY ERUKOVÉ V OLEJI SEMEN *BRASSICA OLERACEA*

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ABSTRACT: Usually, the seed oil of *Brassica oleracea*, i.e., cabbages, cauliflower, kohlrabi, kales, etc., is characterised by a high content of erucic acid (13-docosenoic acid, $C_{22:1}$). However, it has been possible to identify individual plants in accessions of *B. oleracea* conv. *capitata* being nearly free of $C_{22:1}$. Genetic analysis of the progeny has revealed that erucic acid content in this diploid *Brassica* species is under monogenic control and that the low-erucic trait is true-breeding. With regard to the molecular basis of erucic acid genetics our studies support the hypothesis that biosynthesis of $C_{22:1}$ is controlled through the expression of the elongase condensing enzyme (β-ketoacyl-CoA synthase), which is encoded by gene(s) or alleles homologous to the *FAE1* gene of *Arabidopsis thaliana*.

Keywords: *Brassica oleracea*; cabbage; erucic acid; low-erucic mutants; inheritance; *Arabidopsis thaliana*; β-ketoacyl-CoA synthase (KCS)

ABSTRAKT: Olej semen *Brassica oleracea*, tj. zelí, květáku, kedlubnu, kapusty atd., se obvykle vyznačuje vysokým obsahem kyseliny erukové (kyselina 13-docosenová, $C_{22:1}$). Ve vzorcích *B. oleracea* conv. *capitata* však byly nalezeny rostliny téměř bez $C_{22:1}$. Genetická analýza jejich potomstva prokázala, že obsah kyseliny erukové tohoto diploidního druhu je kontrolován jedním genem a že nízký obsah kyseliny erukové je recesivním znakem. Genetické studie obsahu kyseliny erukové na molekulární úrovni podporují hypotézu, že biosyntéza $C_{22:1}$ je kontrolována expresí enzymu kondenzující elongázu (β-ketoacyl-CoA syntáza), který je kódován geny nebo alelami homologickými s genem *FAE1* v *Arabidopsis thaliana*.

Klíčová slova: *Brassica oleracea*; brukev; kyselina eruková; nízkoerukové mutanty; dědičnost; *Arabidopsis thaliana*; β-ketoacyl-CoA syntáza (KCS)

INTRODUCTION

Very long-chain fatty acids (VLCFA) with more than 18 carbons are major components in the seed oil of certain plant families, including *Brassicaceae*, *Tropaeolaceae* and *Limnanthaceae* (Lühs and Friedt, 1994a; Lühs, 1996; Lühs *et al.*, 1999a). Since erucic acid, the dominant fatty acid of *Brassica* seed oils, is considered as detrimental to the food quality, low-erucic acid varieties of *B. napus* (oilseed rape; AACC genome, $2n = 4x = 38$), *B. rapa* (turnip rape; AA genome, $2n = 2x = 20$), *B. juncea* (Brown mustard; AABB genome, $2n = 4x = 36$) and *B. carinata* (Ethiopian mustard; BBCC genome, $2n = 4x = 34$) were successfully bred showing an almost complete abolishment of VLCFA synthesis. Genetic studies have shown that in *B. napus*, which arose from spontaneous crosses between *B. rapa* (syn. *campestris*) and *B. oleracea* (cabbage, cauliflower, Kohlrabi, etc.; CC genome, $2n = 2x = 18$), the $C_{22:1}$ content is controlled by two gene loci, which have additive effects (Stefansson *et al.*, 1961; Downey, 1964; Jönsson, 1977; Kirk and Oram, 1981; Lühs and Friedt, 1994b; Getinet *et al.*, 1994).

With regard to the molecular basis of erucic acid synthesis it is well known that VLCFA are synthesised by the acyl-CoA elongase catalysing the addition of two carbon units from malonyl-CoA to an acyl-CoA similar to the reaction sequence of the plastidial fatty acid synthetase system (Fehling and Mukherjee, 1991; Cassagne *et al.*, 1994; Millar and Kunst, 1997). Among the constituent enzymes of the microsomal fatty acid elongase the β-ketoacyl-CoA synthase (KCS) is considered to catalyse the rate-limiting step determining the acyl chain length of the VLCFA produced in the seed. In the initial step malonyl-CoA condenses with oleoyl($C_{18:1}$)-CoA of a

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cytosolic pool which is maintained by the plastidial fatty acid synthesis (Bao *et al.*, 1998). Subsequently, the condensation product undergoes a reduction, a dehydration and a further reduction step to remove the keto group from the elongated acyl chain. Via a further elongation cycle, eicosenoyl($C_{20:1}$)-CoA is converted to $C_{22:1}$-CoA and becomes available to seed oil synthesis. In addition to the $C_{18:1}$-CoA dependent elongation, an ATP dependent elongation pathway appears to coexist in rapeseed, but it is unclear to which extend it contributes to VLCFA synthesis (Hlousek-Radojcic *et al.*, 1995; Domergue *et al.*, 1999).

Recently, progress in elucidating organisation and control of the VLCFA biosynthetic pathway has been achieved by cloning genes, *FAE1*, *KCS*, *CUT1*, *FIDDLEHEAD* (*FDH*), encoding putative condensing enzymes (Fig. 1), which are expressed in the seed or epidermis of different plants (James *et al.*, 1995; Lassner *et al.*, 1996; Millar and Kunst, 1997; Clemens and Kunst, 1997; Barrett *et al.*, 1998; Fourmann *et al.*, 1998; Han *et al.*, 1998; Millar *et al.*, 1998, 1999; Todd *et al.*, 1999; Venkateswari *et al.*, 1999; Yephremov *et al.*, 1999; Pruitt *et al.*, 2000). Functional expression studies provided strong evidence that the amounts of VLCFAs produced by the different elongase systems are primarily controlled by the expression levels of the KCS genes. In addition, besides the composition of the substrate pool available for elongation, the substrate specificities of the condensing enzymes play a decisive role in determining chain length and degree of unsaturation of the elongation products (Lassner *et al.*, 1996; Millar and Kunst, 1997; Millar *et al.*, 1998).

MATERIAL AND METHODS

Plant material

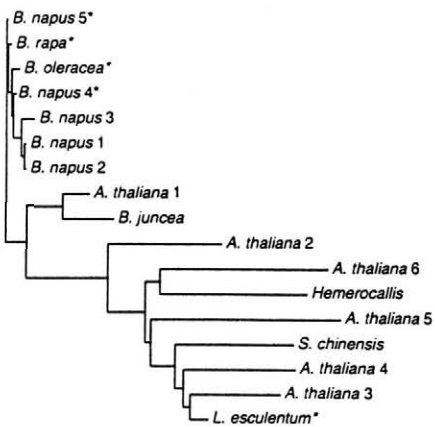
A set of 30 *Brassica* genotypes consisting of 5 *B. oleracea* (genome CC), 5 *B. rapa* (AA), 15 *B. napus* (AACC) and 3 *B. juncea* (AABB) accessions as well as *B. nigra* cv. Giebra (BB) and an Ethiopian *B. carinata* (CCBB) accession were included in this examination. The seed samples were part of our own stocks or were purchased at local seed stores, respectively. Other *Brassica* materials were received from USDA-ARS, Plant Genetic Resources Unit, Cornell University, Geneva, NY, USA. Among the *B. oleracea* genebank accessions Kashirka 202, Ladozhskaya DS 8395 and Eisenkopf individual seeds were identified being very low in erucic acid content. In the latter *B. oleracea* material fatty acid composition was determined on the basis of half-seed analyses as described earlier (Lühs and Friedt, 1994a; Lühs, 1996). The corresponding half-seed plants were cultivated in the greenhouse, artificially vernalised and self-pollinated by bagging. The seed progeny was analysed for fatty acid composition in order to check for true-breeding individuals.

Molecular characterisation

By using a PCR amplified fragment of the *A. thaliana FAE1* gene (James *et al.*, 1995) as probe, a promising *B. napus* cDNA clone (KCSb5) with a fragment size of 1.6kb was isolated from a rapeseed (cv. Askari) silique cDNA library (cf. Han *et al.*, 1998). Genomic DNA from young green leaf tissue was extracted and digested with restriction enzyme *BamH1*. In the course of genomic Southern analysis the KCSb5 gene was used as probe for the detection of erucic acid genes/alleles (Fig. 2, top) according to Lühs *et al.* (1999a, b). PCR amplified fragments generated by deduced primer pairs and covering a range of 501bp (KCSs1/as1), 381bp (KCSs2/as2), and 1251bp (KCSs1/as2) of KCSb5 were separated on a 1.5% agarose gel and visualised by ethidium bromide staining in order to detect polymorphisms among a set of *Brassica* genotypes (Fig. 2, bottom).

RESULTS AND DISCUSSION

Erucic acid content in the seed oil of *Brassica* species varies with the allelic constitution of the genotype, differences in the ploidy level, the genetic background and



* partial cDNA sequence

Fig. 1. Phylogram based on the analysis of the amino acid sequences of the condensing enzyme and putative ones of the following plant species: *B. napus* 4 and 5, *B. rapa*, *B. oleracea* (Fourmann *et al.*, 1998), *B. napus* 3 (Barret *et al.*, 1998), *B. napus* 1 (Han *et al.*, 1998), *B. napus* 2 (Clemens and Kunst, 1997; Barret *et al.*, 1998), *A. thaliana* 1 (James *et al.*, 1995), *B. juncea* (Venkateswari *et al.*, 1999), *A. thaliana* 2 (AL023094), *A. thaliana* 5 (AC004484), *A. thaliana* 6 (AC005818), *Hemerocallis* hybrid (AF082033), *S. chinensis* (Lassner *et al.*, 1996), *A. thaliana* 4 (AC002411), *A. thaliana* 3 (AC003105), *Lycopersicon esculentum* (X83420)

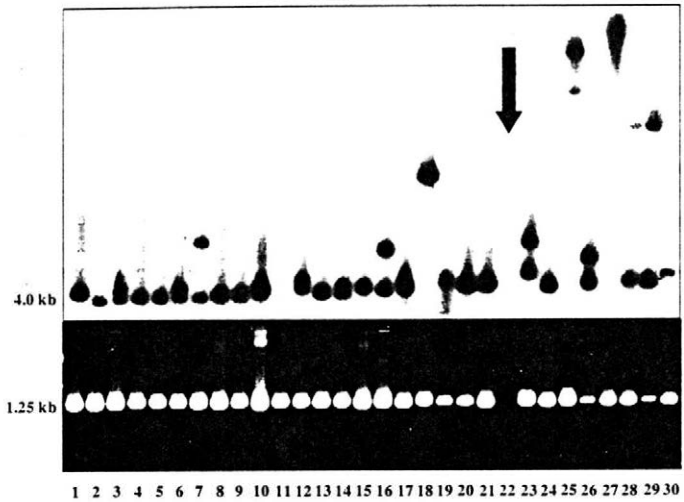


Fig. 2. Molecular characterisation of low-erucic acid mutant (lane 22, arrow) and wild-type *B. oleracea* Ladozhskaya (lane 23) by genomic Southern analysis (top; 4.0 kb fragments) and KCS-specific PCR (bottom; 1.25 kb fragments); The set of 30 *Brassica* genotypes comprises 5 *B. oleracea* (lanes 2, 22–24, 26), 5 *B. rapa* (lanes 1, 18–21), 15 *B. napus* (lanes 3–17) and 3 *B. juncea* (lanes 27–29) accessions as well as *B. nigra* cv. Giebra (lane 25) and an Ethiopian *B. carinata* (lane 30) accession; lanes 11, 25 and 27 non-sufficient DNA quality in Southern analysis (Lühs *et al.*, 1999b)

environmental impact. Series of alleles have been identified in *B. napus* (genome AACC) and *B. rapa* (AA), which make it possible to breed strains containing almost any level of $C_{22:1}$ from less than 0.5% to about 60% of total fatty acids (Jönsson, 1977). However, only in a few cases evidental data is available, which confirms the presence of true alleles residing, for example, either in the A or C genome of *B. napus* (Anand and Downey, 1981; Chen and Heneen, 1989; Lühs and Friedt, 1995b). Contrary to oilseed rape and turnip rape, genetic studies dealing with the fatty acid composition of the seed oil of *B. oleracea* (CC) are lacking. This rather neglected species of the genus *Brassica* normally displays a high $C_{22:1}$ content ranging from 28 to 63% (Lühs and Friedt, 1995a). In the course of experiments developing resynthesised rapeseed Heneen and co-workers were able to demonstrate independent inheritance of erucic acid content and flower colour in the C genome of synthetic *B. napus* (AACC) (Chen *et al.*, 1988).

Identification of low-erucic mutants of *Brassica oleracea*

In the course of studies dealing with the inheritance of erucic acid content in the seed oil of *Brassica* species (Lühs *et al.*, 1999a, b) individual plants belonging to three accessions of *B. oleracea* conv. *capitata*, Kashirka 202, Ladozhskaya DS 8395 and Eisenkopf, were identified being very low in erucic acid content. Applying half-seed selection the subsequent analysis of the progeny might confirm whether erucic acid content of *B. oleracea* is determined by one gene locus, as one could expect, since previous studies indicated an additive mode of inheritance in some *Brassicaceae* species including *B. rapa* (Dorell and Downey, 1964), *B. napus* (Harvey and Downey, 1964; Chen and Heneen, 1989; Lühs and Friedt, 1995b), *B. juncea* (Kirk and Hurlstone, 1983), *B. carinata* (Getinet *et al.*, 1997) and *Sinapis alba* (Raney *et al.*, 1995).

Among 26 seeds of the cabbage Kashirka 202 with an average erucic acid content of 20.9% we were able to select 8 seeds with less than 1% $C_{22:1}$, 17 seeds with a medium level of erucic acid (20.8–34.8%) and a single half seed with 47.2% $C_{22:1}$. Following self-pollination the progeny of the latter plant ($n = 19$ seeds) showed a stable erucic content ranging from 38.5–49.8% (average 44.4%) and indicated that this plant represented a true-breeding high-erucic genotype (E_cE_c). Seeds with a medium erucic acid content were assumed to be heterozygous for the erucic acid locus. Testing of the selfed progeny of a putatively heterozygous half seed plant (27.2% $C_{22:1}$) revealed a similar average erucic acid content of 26.8% and a segregation pattern that fitted well to the expected Mendelian monogenic additive mode of inheritance: 1/4 E_cE_c (42.5–47.0%):2/4 E_ce_c (26.7–33.7%):1/4 e_ce_c (<0.3%). As far as we know, this is the first time, that low-erucic acid mutants of *B. oleracea* have been described and studied with regard to fatty acid inheritance.

Molecular genetics of erucic acid content

The results of genomic Southern analysis (Fig. 2, top) substantiated the molecular basis of $C_{22:1}$ content in the genus *Brassica* showing a distinct variation within KCS alleles. For example, the two parental erucic acid genes of *B. rapa* Yellow Sarson (lane 1) and *B. oleracea* BK 2287 (lane 2) were transferred into the descendant, the synthetic *B. napus* line Res 91 (lane 3). *B. napus* of high- and low-erucic acid type were not generally distinguishable whereas high-erucic acid winter turnip rape Arktus (lane 19) and low-erucic acid spring type *B. rapa* Asko (lane 18) displayed different bands. PCR amplifications with

the KCS-specific primer pairs revealed no polymorphisms between high- and low-erucic genotypes indicating that only a minor change has taken place for mutation into low-erucic acid alleles. However, in the progeny of the *B. oleracea* accession Ladozhskaya DS 8395 a completely different pattern was observed as no signal in the 'selection zero' (lane 22, arrow) of this cabbage was detected indicating partial or complete deletion of the *KCS* gene. The latter result was verified through PCR analysis with a KCS-specific primer pair (Fig. 2, bottom), where the 1.25kb fragment characteristic for all other genotypes was not amplified in the case of the 'zero erucic' mutant (lane 22, arrow). Recently, Roscoe *et al.* (1998) concluded that the mutated gene(s) manifesting the low-erucic acid phenotype act(s) post-transcriptionally. These authors suggest that variation in the sequence of the *FAE1* gene can lead to an apparently inactive protein resulting in deficiency of elongation activity in developing rapeseed (Roscoe *et al.*, 1998). We assume that the low-erucic mutation we have identified in one of the *B. oleracea* genotypes is acting differently and is leading to the absence of a KCS protein. The characterisation of this mutant will be a matter of further investigation.

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INSECT AND FUNGAL ENZYME INHIBITORS IN STUDY OF PLANT VARIABILITY AND EVOLUTION*

INHIBITORY HMYZÍCH A HOUBOVÝCH ENZYMŮ PŘI STUDIU VARIABILITY A EVOLUCE ROSTLIN

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ABSTRACT: Amylase and proteinase inhibitors were found to be highly polymorphic in seeds and vegetative tissues of wild and cultivated species of wheat, rice, other cereals, *Vigna* sp., kidney bean, potato, sunflower and other *Compositae*. The spectra of various inhibitors determined by isoelectric focusing are specific for species, genomes or varieties and reflect evolutionary relationships between species in various plant groups. Potato varieties differed in their capability to accumulate inhibitors in leaves in response to damage and in the spectra of induced inhibitors. Inhibitors can be effectively used in studies of plant diversity, evolution and plant-parasite co-evolution in combination with other protein and DNA markers.

Keywords: trypsin; chymotrypsin; subtilisin; insect α-amylase; cysteine proteinase; inhibitor; fungi; wheat; rice; cereals; potato; vigna; sunflower; safflower; *Compositae*; evolution; diversity

ABSTRAKT: Inhibitory amylázy a proteinázy v semenech a vegetativních pletivech planých i pěstovaných druhů pšenice, rýže, jiných obilnin, druhů *Vigna* sp., fazolu, slunečnice a jiných hvězdnicovitých jsou vysoce polymorfní. Spektra různých inhibitorů, stanovená izoelektrickým fokusováním, jsou specifická pro druhy, genomy nebo odrůdy a odrážejí evoluční vztahy mezi druhy v různých botanických uskupeních. Odrůdy bramboru se lišily ve schopnosti akumulovat inhibitory v listech při reakci na poškození a ve spektrech indukovaných inhibitorů. Inhibitory mohou být účinně využívány v kombinaci s jinými proteiny a markery DNA ke studiu diverzity rostlin, jejich evoluce a koevoluce s parazity.

Klíčová slova: trypsin; chymotrypsin; subtilizín; hmyzí α-amyláza; cysteinová proteináza; inhibitor; houby; pšenice; rýže; obilniny; brambor; *Vigna*; slunečnice; saflor; hvězdnicovité; evoluce; diverzita

INTRODUCTION

The seeds and vegetative parts of higher plants contain various proteinaceous inhibitors of insect, fungal, mammalian and endogenous proteinases. The inhibitors may be involved in plant defense systems against harmful organisms and may also play regulatory roles during plant development (Shewry and Lucas, 1997). Furthermore, plant inhibitors are of interest in relation to problems of host/parasite co-evolution (Konarev, 1996), as markers in studies of plant diversity and evolution (Konarev, 1982, 1996) and as potential drugs with antiviral and other properties. Genes encoding potent and stable inhibitors can be transferred to other plants to improve their pest or fungal resistance (Ryan, 1990). The biochemical properties of hydrolase inhibitors are particularly well studied in the families *Fabaceae*, *Poaceae* and *Solanaceae*. Some 12 inhibitor families can be recognised based on their amino acid sequences and target proteinases (Shewry, 1999). However, the evolutionary variability of inhibitors in these taxa was not investigated in detail. The inhibitors of sunflower and other species of the *Compositae* remained unstudied until recently. We have, therefore, (i) determined the polymorphism, distribution, variability, genetic control and biochemical properties of α-amylase and proteinase inhibitors in lines, varieties

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and wild accessions of wheat, *Aegilops*, rice, potato, cowpea, kidney bean, sunflower and other *Compositae* species and (ii) searched for novel inhibitors. Special attention has been given to inhibitors of insect α-amylases and cysteine proteinases, trypsin, which is a typical digestive enzyme of insects, mammals and fungi, and subtilisin, a proteinase of microorganisms.

MATERIAL AND METHODS

Seed material was obtained from the Vavilov Institute of Plant Industry (St. Petersburg). Most of the study was carried out using simple and sensitive methods to detect amylase (Konarev, 1982; Konarev and Fomicheva, 1991) and proteinase (Konarev, 1986; Konarev *et al.*, 1999b, 2000a) inhibitors among other plant proteins, separated by isoelectric focusing (IEF), electrophoresis or thin layer gel-filtration. These methods were also effective in monitoring stages of purification of novel inhibitors by gel-filtration, affinity chromatography and reversed-phase HPLC.

RESULTS AND DISCUSSION

Cereals. In wheat (*Triticum* L.) and *Aegilops* L. the spectra of insect amylase and proteinase inhibitors determined by IEF are specific for species, genomes or varieties and reflect evolutionary relationships between diploids and polyploids (Konarev, 1982, 1996). The polymorphism of inhibitors of chymotrypsin and subtilisin (C/SI) is comparable with that of prolamins and can be used in wheat variety identification.

In rice, inhibitors of trypsin, subtilisin and insect α-amylases were found in accessions of *Oryzae sativa* and other diploid species with the A genome but not in diploid species with the B and C genomes or tetraploid species with the BBCC and CCDD genomes. They therefore reflect divergence between rice species with A and other genomes.

Fabaceae. Data for four serine and cysteine proteinase inhibitor systems allowed evolutionary links between *Vigna* species to be estimated and identified diploid forms that may be related to the donors of the genomes of tetraploids. Data on 74 inhibitor band positions were in good agreement with data on morphology and DNA analysis and, in addition provided new information on the evolutionary relationships between species of the subgenus *Ceratotropis* (Konarev *et al.*, 1999c, 2000b).

Potato. *Solanum tuberosum* varieties differed in the spectra of proteinase inhibitors present in tubers, in their ability to accumulate inhibitors in leaves in response to damage and in the spectra of induced inhibitors. Ability to accumulate inhibitors was found only among varieties resistant to Colorado beetle (Konarev and Fasulati, 2000).

Compositae. Multiple molecular forms of proteinaceous inhibitors of digestive proteinases of animals and extracellular proteinases of phytopathogenic fungi were identified in seeds of wild and cultivated *Helianthus* and other *Compositae* species. *H. annuus* seeds contain at least two types of trypsin inhibitors (TI) and bifunctional trypsin/subtilisin inhibitors (T/SI). The main TI, characteristic of the majority of *Helianthus* and some *Tithonia* species, is unique among plant proteinase inhibitors in its small size (1500) and cyclic structure (Konarev *et al.*, 1999a, 2000a; Luckett *et al.*, 1999). The TIs and T/SIs vary widely between *H. annuus* lines and wild *Helianthus* species in their presence or absence, composition, hydrophobicity and pI. Analysis of F_2 hybrids indicated that three loci encoding T/SI components were linked (Konarev *et al.*, 1999a, 2000a). Similar components were found in annual diploid species with the B genome but not in perennials with the A genome. The T/SI present in seeds and vegetative organs were active against extracellular proteinases of the white rot fungus *Sclerotinia sclerotiorum*, an important pathogen of sunflower, indicating a possible protective role (Konarev *et al.*, 1999b). T/SI, C/SI and T/C/SI with mass near 7500 are widely distributed in other *Compositae* species, being present in species of the subfamilies *Carduoideae* (genera *Carthamus*, *Centaurea*, *Cirsium*), *Cichorioideae* (*Lactuca*, *Taraxacum*) and *Asteroideae* (genera *Cosmos*, *Dahlia*, *Bidens*) etc.

Thus, in many plant taxa the spectra of various inhibitors can be specific for lines, varieties, single biotypes, species, groups of species, genomes and genera. Spectra of inhibitors reflect evolutionary links between species within a subgenus or genus or between diploid and polyploid species. The use of inhibitors as markers of plant resistance to harmful organisms can be based on their biological activity or on their physiological or genetic linkage with resistance factors. Methods of detection of amylase and proteinase inhibitors are applicable to representatives of many plant families and can be used to search for novel inhibitor types. Inhibitors can be effectively used in studies of plant diversity, evolution and plant-parasite co-evolution in combination with other protein and DNA markers.

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SURVEYING PLANT METABOLISM BY SEQUENCING ANALYSIS OF EXPRESSED CLONES IN *TRITICEAE**

ZÍSKÁNÍ PŘEHLEDU ROSTLINNÉHO METABOLISMU POMOCÍ SEKVENČNÍ ANALYZY EXPRIMOVANÝCH KLONŮ U *TRITICEAE*

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ABSTRACT: Genomic research gives the opportunity to understand genome structure, gene function and its evolution. ESTs (Expressed Sequence Tags) databases are now produced for several plant species, given the remarkable potential of the technique and its possible applications. In this study an EST database has been developed from a barley cold-induced cDNA library (cultivar Nure) as part of the ITEC (International Triticeae EST Cooperative) project.

Keywords: EST (Expressed Sequence Tags); barley; *Triticeae*; cDNA library; cold acclimation

ABSTRAKT: Výzkum genomu umožňuje pochopit strukturu genomu, funkci genů a evoluci genomu. Databáze značkových exprimovaných sekvencí (Expressed Sequence Tags, ESTs) jsou nyní vytvářeny pro několik rostlinných druhů, neboť tato technika má pozoruhodný potenciál a množství aplikací. Předmětem této studie bylo vytvoření databáze EST z knihovny ječmenné cDNA indukované chladem (odrůda Nure) jako část projektu ITEC (International Triticeae EST Cooperative).

Klíčová slova: EST (Expressed Sequence Tags); ječmen; *Triticeae*; knihovna cDNA; otužování chladem

Automatic high-troughput sequencing methods developed in recent years have provided plant genetists of an invaluable tool for the exploration of the genomes. At present, genomic methods that examine mRNA and proteins will offer insights into mRNA expression, protein expression, protein localization and interactions (Brent, 2000).

A rapid and cost effective way to identify new genes has been developed in a number of laboratories with the systemic sequencing of anonymous cDNAs. In fact, single-run sequences of 250–400 bases (Expressed Sequence Tags, ESTs) on cDNA clones provide useful information to identify corresponding genes and to assign a potential function (Cooke *et al.*, 1996) and of consequence to isolate novel genes. Expressed Sequence Tags databases are now accumulating not only for model plants, but even for several plant species of agronomic relevance, given the remarkable potential of the technique and its possible applications.

At the 9th International Wheat Genetics Symposium held at the University of Saskatchewan in 1998 a proposal was developed to establish a public database of at least 20 000 ESTs from *Triticeae* before July 2000. This initiative was named ITEC, International Triticeae EST Cooperative and was open to all laboratories, with the only restriction to submit a minimum number of EST from *Triticeae*. This activity was seen as the first step in developing a public set of information and materials for *Triticeae* genome research (http://wheat.pw.usda.gov/genome). The ITEC participants have contributed to the common database submitting tags from cDNA libraries from different species of *Triticeae,* subjected to different conditions of growth, from different developmental stages and from different tissues. Fig. 1 reports an overview of the cDNA libraries utilized by ITEC participants, considering the species, the tissue and the environmental conditions from which mRNAs were extracted. Among tissues, leaf and root has been the most frequently utilized for cDNA library construction, and among species the best represented are bread wheat and barley, with genome sizes ranging from 5 000 to more than 16 000 Mb. The applied abiotic stress conditions refer to cold, drought, heat, heavy metals and to disease challenge ranging from virus to fungi. Several developmental stages were considered and the genotypes are most frequently cultivars and lines widely utilized for genetic studies. Most of the libraries are oriented and constructed in phase. Single-run sequences of at least 300 bp with a

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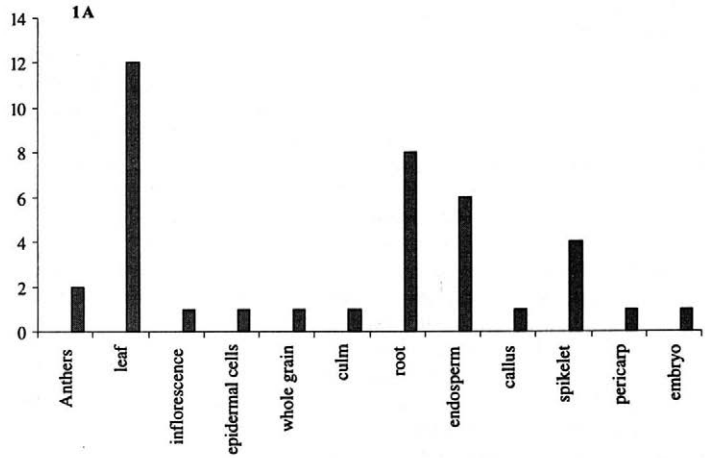
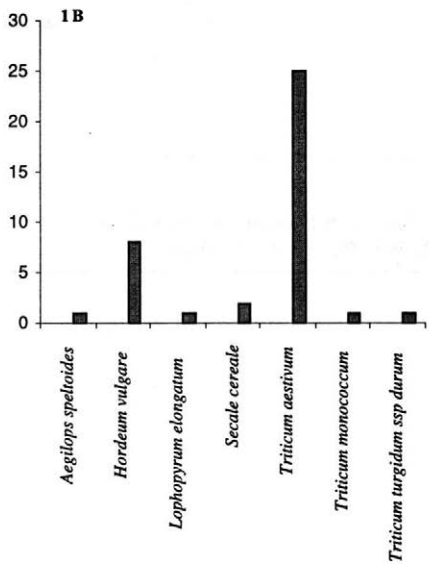
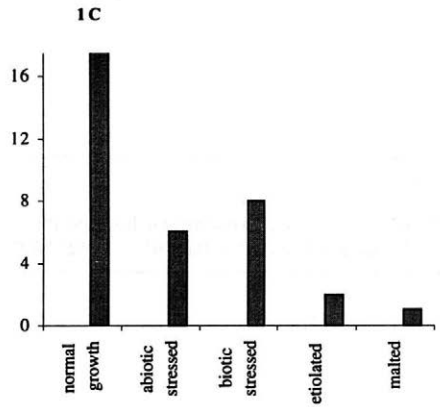


Fig. 1. Some characteristics of the cDNA libraries utilized by ITEC partecipants (1A – tissue; 1B – species; 1C – conditions)





maximum of 5% ambiguous bases have been determined on random clones from unamplified or prescreened cDNA libraries.

As our contribution to the ITEC project, an EST database has been developed from a barley cold acclimated cDNA library (Faccioli *et al.*, submitted). The primary target of the work was to survey the mRNA population in a specific tissue under specific conditions. The second target was the identification of novel genes specifically induced by the applied stress conditions. The cv. Nure has been chosen, a feeding winter barley with good cold tolerance, released in 1998 by the Istituto Sperimentale per la Cerealicoltura, Sez. Fiorenzuola d'Arda. At the first leaf stage the plants were cold acclimated with a thermal treatment of 3°C for 4 days, that has been demonstrated to induce cold acclimation in barley (Cattivelli *et al.*, in

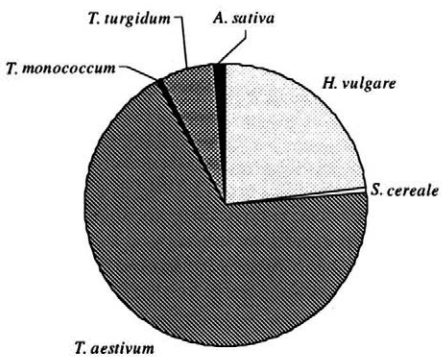


Fig. 2. Sequences present in the ITEC database

press). After cloning work, 960 selected tags have been sequenced and BLAST search (Altschul *et al.*, 1997) was performed against the international nucleotide and protein data banks (EMBL, GenBank, DDBJ, PDB, PIR, SwissProt, GenPept).

A total of 24,544 *Triticeae* EST was sent by different laboratories partecipating to the Cooperative and are now publicy available. Fig. 2 illustrates the contribution of different species to the project: about 68.6 % of the EST are from *Triticum aestivum*, 0.4% from *Triticum monococcum*, 6.1% from *Triticum turgidum* spp. *durum*, 22.6% from *Hordeum vulgare*, 0.4% from *Secale cereale* and 1.3% from *Avena sativa*.

Our present expansions of the EST project are related with the participation in the ITEC database and its use, implementation of our EST database, search for SSR sequences, mapping ESTs on Nure X Tremois DH population map and preparation of cDNA microarrays to survey gene expression in cold and drought conditions.

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FROM THE SPHERE OF SCIENCE

From the 6th International Wheat Conference in Budapest (5–9 June 2000)

The opening of the conference remembered the impact of the rediscovery of Mendel's laws on crop breeding in general and wheat breeding in particular. Prof. M. S. Swaminathan (UNESCO Chair in Ecotechnology) evaluated that Mendelian genetics during the past 100 years helped not only to exploit naturally occurring genetic variability, but also accelerated the process of generation, manipulation and combination of new variability. He divided the Mendelian century into 4 phases:

1. 1900–1930 – early days of Mendelian genetics;
2. 1930–1960 – Mendelian genetics was enlarging the base of the theory and its application;
3. 1960–1980 – quantum jump in wheat productivity, titled "green revolution" brought in by semi-dwarf wheat varieties originating from the CIMMYT breeding programme in Mexico headed by the Nobel prize Laureate Norman Borlaug. The "green revolution" helped to raise public wareness of the role of science in wheat production;
4. 1980 onwards – the "gene revolution" as the integration of Mendelian to molecular genetics. In this period it is very important to find an appropriate blend of Mendelian and molecular breeding. Also in this time the "breeders eye" for selection and for spotting the winner will continue to play an important role in successful plant breeding.

S. Rajaram (CIMMYT Mexico) outlined new approaches for future wheat breeding: re-structuring the wheat plant following the model of "super wheat" with larger spikes and more grains, research and breeding of hybrid wheat, wheats with multiple disease resistance, marker assisted selection, the use of physiological traits as selection criteria. He accented, that investments to pre-breeding are necessary.

The importance of wheat diseases are changing due to new husbandry and cropping systems. "Triple rusts" resistance is still highly required, but septorioses, fusarioses and viroses are becoming more and more dangerous. Molecular markers are expected to have increasing impact on the ability to select gene combinations needed to extend the durability of resistance. M. Henry presented the use of molecular markers in selecting for resistance to BYDV (a simple sequence repeat [SSR] marker gwm37). The importance of using wild progenitors in wheat pre-breeding for disease resistance and also abiotic stress tolerance was presented by J. Valkoun (ICARDA).

Much attention was paid to wheat quality for the end-user. Research results enable us to understand more the genetic and biochemical basis of bread making quality. Shewry P. R. *et al.* reported the use of genetic engineering for the exploration of the mechanism of gluten elasticity and for the achievement of improvements, by means of inserting genes encoding mutant and wild type HMW subunits into model lines and cultivars. The problems with allergies and intolerance to wheat gluten may be solved in future by gene manipulations. Many new micro tests and equipments were presented, which permit to evaluate wheat quality in early breeding generations (F_3).

J. W. Snape stressed the importance of detailed knowledge of the genetic control of key groups of genes controlling the life-cycle duration in wheat, namely those controlling vernalization response, photoperiod response and developmental rate (earliness per se = *Eps* genes).

P. Heffer (FIS/ASSINSEL) described the situation on the wheat seed market and trends in wheat seed research. During the last decades wheat production benefited from modern plant breeding and seed production technologies. 1300 bread wheat and 300 durum wheat varieties are listed on the OECD list. In the world 2500 improved wheat varieties are available. Hybrid varieties were released in 6 countries, but their acreage is low. In France 10 hybrid wheats are registered, they occupy, however, only 3% of the wheat acreage. In Germany one hybrid cultivar is released, which covers 1% of the wheat acreage. Transgenic varieties could be affected by new regulations on GMOs, such as the Biosafety Protocol or compulsory labelling of food containing GMOs. Transgenic wheat varieties may be on the market in the year 2003/2004.

Many presentations mentioned substantially increasing demands on plant breeding, to produce varieties for feeding the growing population of the world. S. Rajaram stressed, that the grassland area per person decreased from 0.19 ha in 1970 to 0.11ha in 1994. Globally speaking, the world would require one billion metric tons of wheat in the year 2020. The current global average wheat yield of 2.5 t/ha must be raised to roughly 4 t/ha in 2020, that is 2.5% annual yield increase. To achieve this goal investments in agricultural science and especially in plant breeding are necessary.

Ing. A. Hanišová, Ing. P. Horčička

AN OBITUARY NOTICE

Dr. Antonín Fojtík, renowned breeder of forage crops, 1934–2000

On September 29, 2000 died in age of 66 years Dr. Antonín F o j t í k, breeder of forage crops of international reputation from Hladké Životice, who was a hard working, helpful and kind person and member of our publishing board.

He was born on November 17, 1934, in Starojícká Lhota. After graduation at the University he was an active plant breeder since 1960 at the Plant Breeding Station Hladké Životice, which he had established together with Dr. Vladimír Světlík, where he also has built a modern cytologic laboratory. He was very successful above all in breeding of tetraploid varieties of grasses and of red clover. In his later breeding work he concentrated on interspecific and intergeneric hybridisation of grasses. His intergeneric hybrids of *Lolium* and *Festuca* are spread also in several European countries, where they are appreciated for their ability to grow also in the winter, which is a prerequisit to keeping cattle outdoors grazing throughout the whole year.

A. Fojtík cooperated closely with colleague breeders from Czechia, Slovakia and other countries, mainly Poland and Germany, and with universities and research institutions. Results of his research and breeding were published in scientific journals and presented on scientific meetings at home and abroad. The success of his breeding work is expressed in 25 registered varieties of forage crops, including four varieties of tetraploid red clover and 21 varieties of grasses seven of which were tetraploid. Dr. Fojtík was therefore respected as a renown expert for methodology in breeding and forage research. Due to his knowledge and experience he became a respected member of breeders, research, methodological and editorial boards, and an appreciated reviewer of publications and dissertations. He always helped and passed his experience to younger colleagues, including the junior author of this memorial.

After the *"velvet" revolution* in 1989 he became, as the main breeder of the Plant Breeding Station, member of the board of *The Czech and Moravian Society of Plant Breeders* and chairman of its forage crops section. It must be appreciated, that after his retirement, he continued his breeding activities and has done valuable work concerning international evaluation of varieties, in spite of already severe health problems in the last years.

We miss him very much.

V. Světlík, O. Chloupek

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The author is fully responsible for the originality of his paper, for its subject and formal correctness. The author shall make a written declaration that his paper has not been published in any other information source.

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If any abbreviation is used in the paper, it is necessary to mention its full form at least once to avoid misunderstanding. The abbreviations should not be used in the title of the paper nor in the summary.

The **title** of the paper shall not exceed 85 strokes. Subtitles of the papers are not allowed either.

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Introduction has to present the main reasons why the study was conducted, and the circumstances of the studied problems should be described in a very brief form.

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