

# ADVANCES IN PLANT BREEDING & BIOTECHNOLOGY TECHNIQUES

BOOK OF ABSTRACTS



Pannonian Plant Biotechnology Association Conference  
for PhD Students in Plant Biology



ADVANCES IN  
PLANT BREEDING &  
BIOTECHNOLOGY  
TECHNIQUES

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The aim of the conference for PhD students working in the field of plant biology is to exchange their ideas and their research work which is the basic for our future. PhD students are invited from all Doctoral Schools of the Universities for participation and presentations of their studies.

# CONTENTS

## ORAL PRESENTATIONS

DOMESTICATION BOTTLENECK AND GENETIC VARIETY OF GLUTENIN GENES IN WHEAT <i>S. Makai, L. Tamás, F. Békés, A. Juhász</i>	10
MOLECULAR BACKGROUND OF SPELT ( <i>T. SPELTA</i> ) PROTEINS AND THEIR ALLERGEN CHARACTERISTICS <i>A. Kovács, S. Tömösközi, F. Békés, L. Láng, A. Juhász, Z. Bedő</i>	12
PROTEOMIC ANALYSIS OF ALLERGENIC PROTEINS IN WHEAT <i>S. Fekecsová, M. Hajdúch</i>	14
THE GENES OF AN ORGAN DEVELOPMENT REGULATING TRANSCRIPTION FACTOR FAMILY IN <i>BRACHYPODIUM DISTACHYON</i> <i>M. Gombos, Z. Zombori, M. Szécsényi, Gy. Sándor, J. Györgyey</i>	16
THE EFFECTS OF WHEAT GLUTENIN SUBUNITS ON THE FUNCTIONAL PROPERTIES OF RICE DOUGH <i>S. Pólya, G. Balázs, S. Tömösközi, F. Békés, L. Tamás, M. Oszvald</i>	19
“GLOBALIZATION” IN THE SEXUAL REPRODUCTION OF CEREALS <i>D. Polgári, L. Sági</i>	22
IDENTIFICATION OF MITOGEN-ACTIVATED PROTEIN KINASE SUBSTRATES IN ARABIDOPSIS PROTOPLAST TRANSIENT EXPRESSION SYSTEM <i>M. Dóry, Z. Doleschall, H. Ambrus, R. Dóczi</i>	24
REGULATION OF PROTECTIVE PROLINE SYNTHESIS IN <i>ARABIDOPSIS THALIANA</i> <i>D. Aleksza, H. Kovács, L. Szabados, G. V. Horváth</i>	26
FUNCTIONAL ANALYSIS OF CANDIDATE VIRULENCE GENES OF PHYTOPATHOGEN FUNGUS <i>VERTICILLIUM ALBO-ATRUM</i> PREDICTED BY FUNGAL COMPARATIVE GENOMICS AND PROTEOMIC ANALYSIS <i>M. Flajšman, J. Jakše, S. Mandelc, S. Radišek, B. Javornik</i>	29
TRICHOMES TYPES AND THEIR DENSITY IN PARENTAL SPECIES <i>SOLANUM TUBEROSUM</i> AND <i>S. CHACOENSE</i> AND THEIR DERIVED SOMATIC HYBRIDS <i>A.-M. Mărgineanu, I. Molnár, E. Rakosy -Tican</i>	32

THE EVALUATION OF RESISTANCE TO COLORADO POTATO BEETLE AND DETECTION OF GLYCOALKALOID CONTENT BY USING FOURIER TRANSFORM INFRARED SPECTROSCOPY IN <i>SOLANUM TUBEROSUM</i> + <i>SOLANUM CHACOENSE</i> SOMATIC HYBRIDS	34
<i>I. Molnár, R. Boitor, R. Thieme, T. Thieme, E. Rakosy-Tican</i>	
BIOPHYSICAL CHARACTERISATION AND <i>IN VITRO</i> DIGESTIBILITY OF SUNFLOWER STORAGE PROTEINS	36
<i>B. Berecz, C. Mills, F. Láng, L. Tamás, P. Shewry, A. Mackie</i>	
PROTEOMIC ANALYSIS OF FLAX ADAPTATION IN CHERNOBYL AREA USING GEL-FREE AND GEL-BASED APPROACH	38
<i>D. Gábrišová, M. Danchenko, M. Hajduch</i>	
RECENT ADVANCES IN STYRIAN OIL PUMPKIN BREEDING	40
<i>K. Košmrlj, J. Murovec, D. Kastelec, A. Kladnik, B. Bohanec</i>	
THE EVALUATION OF PROLINE, PLANT REGENERATION AND VIABILITY AFTER <i>IN VITRO</i> STRESS SELECTION SUSTAINS DROUGHT RESISTANCE IN POTATO MARKER-FREE TRANSGENIC LINES CARRYING PVYCP INTRON CONTAINING HAIRPIN CONSTRUCT	43
<i>R. Mustata, I. Molnár, E. Rákosy-Tican</i>	
TRANSGENIC PLANTS BENEFIT FROM REACTIVE ALDEHYDE DETOXIFYING AND SUGAR ALCOHOL PRODUCING FUNCTION OF THE SAME ALDO-KETO REDUCTASE ENZYME DURING HEAVY METAL, FREEZING AND SALT STRESS	45
<i>Cs. Éva, H. Zelenyánszki, R. Tömösközi-Farkas, Á. Solti, L. Tamás</i>	
EFFECT OF THE HARDENING UNDER DIFFERENT LIGHT CONDITIONS ON MAIZE CHILLING TOLERANCE	47
<i>O. K. Gondor, M. Pál, T. Janda, G. Szalai</i>	
THE EFFECTS OF DROUGHT ON PLANT DEFENCE SYSTEM IN WHEAT GENOTYPES WITH DIFFERENT SALICYLIC ACID CONTENT	49
<i>V. Kovács, O. K. Gondor, I. Majláth, G. Szalai, T. Janda, M. Pál</i>	
SOLID-STATE FERMENTATION – AN ALTERNATIVE METHOD FOR ENRICHMENT OF AGRO-BASED MATERIALS WITH BIOACTIVE COMPOUNDS	51
<i>L. Guothová, V. Matušovicová, T. Klemková, M. Čertík</i>	
COLD RESPONSE AND GENE EXPRESSION PATTERN CHANGES IN TWO CHROMOSOME SUBSTITUTION LINES OF WHEAT ( <i>TRITICUM AESTIVUM</i> L.)	54
<i>B. Kalapos, G. Galiba, F. Marincs</i>	

UTILIZATION OF DIPLOID WHEATGRASSES FOR ALIEN CHROMOSOME DISCRIMINATION IN WHEAT × AGROPYRON GLAEL HYBRID PROGENIES	56
<i>K. Kruppa, É. Szakács, M. Molnár-Láng</i>	
NOVEL UTILIZATION OF WILD RELATIVES IN BREAD WHEAT PREBREEDING	59
<i>P. Mikó, M. Megyeri, M. Molnár-Láng</i>	
DEVELOPMENT AND IDENTIFICATION OF NEW WHEAT-BARLEY DITELOSOMIC ADDITION LINES USING FLUORESCENCE <i>IN SITU</i> HYBRIDIZATION AND MOLECULAR MARKERS	62
<i>E. Türkösi, A. Cseh, M. Molnár-Láng</i>	
RESTRICTION FRAGMENT LENGTH POLYMORPHISM MAPPING OF FLAX GROWN IN RADIO-CONTAMINATED CHERNOBYL ENVIRONMENT SUGGESTS THE STABILITY OF DELTA-12 DESATURASE AND FATTY ACID DESATURASE 3C GENES	64
<i>V. Lancíková, J. Žiarovská, M. Danchenko, V. Berezna, M. Bežo, K. Ražná, N. M. Rashydov, M. Hajduch</i>	
STRIGOLACTONES AND PARASITIC WEEDS	66
<i>R. Matusova</i>	
MOLECULAR DIVERSITY AND PHYLOGEOGRAPHY OF BEGOMOVIRUSES INFECTING VEGETABLE CROPS IN INDIA	68
<i>B. S. Bhatt, A. K. Singh</i>	
ANALYSIS OF NSS PROTEIN OF HUNGARIAN RESISTANCE-BREAKING TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATES FROM PEPPER	70
<i>Zs. Csömör, A. Almási, K. Nemes, K. Salánki, G. Csilléry, L. Palkovics, I. Tóbiás</i>	
EFFECT OF MICROALGAE LEAF TREATMENTS ON SUNFLOWER GROWTH AND PRODUCTION	73
<i>P. Póthe, I. Gergely, V. Ördög</i>	
EFFECT ON THE GROWTH, CONDITION, NUMBER OF QUALITY AND OTHER PARAMETERS OF PEPPER KALDOM BY USING NOSTOC ENTHOPHYTUM MICROALGAE-TREATMENT	76
<i>J. Tóth, V. Ördög</i>	

## POSTERS

- THE PLOIDY AND GENOME COMPOSITION OF SOLANUM TUBEROSUM AND SOLANUM BULBOCASTANUM SOMATIC HYBRIDS AND THEIR DERIVED BACKCROSS PROGENIES** 80  
*T.-É. Dénes, I. Molnár, K. Kruppa, É. Szakács, M. Molnár-Láng, R. Thieme, E. Rákósy-Tican*
- VARIAMETRIC ANALYSIS OF EINKORN (TRITICUM M. MONOCOCCUM) SEED POPULATIONS: MEASURING GENETIC DISTANCE USING PHENOVIARIATION** 82  
*A. Emődi, F. Gyulai, Z. Mravcsik, G. Gyulai, M. Megyeri, S. Vinogradov, T.A. Szabó, I. Rovner*
- PRODUCING WHEAT/BARLEY INTROGRESSION LINES USING THE 2C GAMETOCID SYSTEM** 85  
*D. Icsó, G. Linc, M. Molnár-Láng*
- BIOTECHNOLOGICAL APPROACH TO CONTROL PARASITIC WEEDS** 87  
*D. Kullačová, R. Matusova*
- MOLECULAR CHARACTERIZATION OF NOSTOC AND ANABAENA MICROALGAE ISOLATES** 89  
*N. Makra, G. Gell, A. Juhász, V. Soós, Z. Molnár, V. Ördög, E. Balázs*
- GENOTYPE IDENTIFICATION OF ANCIENT VINEGRAPE (VITIS V. VINIFERA) SEED REMAINS (15 CENT. HUNGARY) BY DIGITAL MORPHOMETRY** 91  
*Z. Mravcsik, F. Gyulai, A. Emődi, G. Gyulai, S. Vinogradov, I. Rovner*
- LIGHT QUALITY INFLUENCES THE EXPRESSION LEVEL OF SOME CBF GENES** 93  
*A. Novák, Á. Boldizsár, B. Tóth, G. Galiba*
- SIGNIFICANTLY INCREASED GLUTATHIONE-S-TRANSFERASE ACTIVITY IN 35S (CAMV)-ZMGSTF4 TRANSGENIC ARABIDOPSIS THALIANA** 95  
*K. Pilinszky, A. Bittsánszky, G. Gyulai, T. Kőmíves*



# ORAL PRESENTATIONS

# DOMESTICATION BOTTLENECK AND GENETIC VARIETY OF GLUTENIN GENES IN WHEAT

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Hexaploid wheat only exists in cultivated form and it is derived from a cross between the cultivated *Triticum dicoccum* and a wild goat grass. In one possible scenario its progenitors were as follows: *Triticum urartu* – A genome, *Aegilops speltoides* B – genome and *Aegilops tauschii* – D genome.

The progenitors of wheat acquired many morphological and physiological novelties, such as loss of seed shattering, increased yield, decreased chemical and morphological defenses, and uniformity in germination and growth phenology, erect growth to facilitate increased plant density in crop fields. All these are referred as domestication syndrome. Further traits were acquired during the cultivation process such as diversification in grain starch composition, and adaptation to different climates and latitudes and decrease in grain protein to carbohydrate ratio. Amongst the domesticated crops only hexaploid wheat went through speciation, all other plants retained their genetic relations to their wild types.

Domesticated, hexaploid wheat has unique bread-making quality amongst the members of the *Triticeae* tribe. During the preparation of the bread dough, wheat flour is mixed with water that forms the gluten matrix, a giant, elastic and extensible polymer where the composing proteins are bound by intra- and intermolecular disulfide bridges. The main components of wheat flour are high molecular weight glutenin, low molecular weight glutenin subunits (HMW GS and LMW GS), alpha-, gamma- and omega gliadins and starch. The ratio of prolamins to carbohydrates and the quality and composition of these prolamins in the seed directly influence the end-use quality; consequently all these properties have been greatly affected by the domestication event and the subsequent cultivation.

One detrimental consequence of domestication is the decreased genetic diversity of genes related to domestication syndromes. This genetic restriction, also called genetic bottleneck, may restrict the possibilities of the breeder but can be overcome by backcrossing, as wild type plants present an unchartered genetic depositary. Indeed, haplotype analysis of *Glu-1D* genes reported less genetic diversity than their wild counterparts which is mainly due to the genetic bottleneck caused by human selection processes.

Prolamins are specially expressed in the endosperm of the seed and are regulated via *cis*-regulatory elements. HMW GS proteins are encoded in the *Glu-1* locus on the chromosome 1 of the three homoeolog genomes of hexaploid wheat. Due to an

ancient duplication event at *Glu-1* locus, each locus contains two paralog glutenin genes, named as x and y type genes.

Our earlier study reported that the promoters of *Glu-1* genes have a conserved structure of 7 cis-regulatory modules (CRM) including the proximal basal promoter region. The motif composition of these modules varies across the x and y pairs as well as across the homeolog genes causing variation of activity between these genes. There are distinct differences between the modules of x and y type genes. We concluded that breeding at large did not influence the promoter architecture but rather the activity of the interacting transcription factors. In this study, we aim to analyze the role of domestication bottleneck that may have resulted the conserved, non-optimal (from the breeder's aspect) differences of promoter motif composition of the paralog *Glu-1* genes.

The analysis involved 156 promoter sequences of HMW GS from the *Triticeae* tribe. It contained 69 x-type and 76 y-type sequences. The modules of the promoter sequences were compared across species. Where information was available, the coding region were analyzed for their conserved features.

The expression of x and y type HMW GS genes are different, x type genes always are expressed in higher amounts than their paralogs. In the case of A genome, y-type gene is generally silent in the hexaploid wheat. This can be explained either as a result of the breeding process or a simple heritage of the domestication events leading to hexaploid wheat. In case of the former, the stoichiometric ratio of x and y-type genes may represent an optimum for the end-use quality, thus it was the target of the selection process. This is supported by the fact that the numbers of cysteine and their distribution on the protein are characteristically different for x and y-type HMW GS. Since these cysteine residues play a crucial role in the development of the gluten matrix this aspect is not negligible, although needs a thorough examination.

The other cause of the different expression level may lie in the fact that polyploidization is a relatively rare event. The diversity of HMW-GS genes were determined at this moment of speciation that represented a genetic bottleneck that narrowed the gene pool of the new specie. In this case, breeding could not broaden the diversity beyond a certain degree, thus we experienced low variability. This is supported by the fact that the diversity of wild type genes is higher than in the case of hexaploid wheat for both the cysteine distribution and promoter motif composition. However, there were more than one polyploidization event that lead to hexaploid wheat. First, the merge of the A and B genomes, then the merge of this tetraploid with the D genome. It seems very likely that the above described two possibilities both were at place but with different weight in these separate events.

## ACKNOWLEDGEMENT

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# MOLECULAR BACKGROUND OF SPELT (*T. SPELTA*) PROTEINS AND THEIR ALLERGEN CHARACTERISTICS

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Wheat is one of those food components that are responsible for a large amount of food hypersensitivity reactions. There are a several type of wheat-related hypersensitivity (baker's asthma, IBS: irritable bowel syndrome, gluten intolerance). The most widespread diseases are wheat allergy and celiac disease triggered by certain proteins and their given short peptide sequences (epitopes) in wheat and a few other cereals (barley, rye, oat and triticale). The initial clinical symptoms of these disorders are often overlapping, that makes their differentiation and diagnosis difficult, however their pathogen mechanisms are absolutely different. The wheat allergy is an IgE-mediated reaction, pathological reaction of the immune system to wheat proteins. The celiac disease is an autoimmune disorders to prolamins (gluten proteins) of cereals. IgA/IgG are generated through the immune response. The prevalence of the celiac disease in the Caucasoid (Europid) race (inclusive of Hungary) is not more than 2%, until the rate of the population affected by any kind of cereal-related sensitivity/allergy with different severity and mechanism is more than 4%.

In case of both disorders the information about the target molecules (antigens) are incomplete. The classical separation method of the wheat proteins is the Osborne solvent fractionation procedure. According to this alignment the water-soluble components are the albumins, the salt-soluble are the globulins, the prolamins, which are soluble in alcohol and the glutenins, which are only soluble in dilute acids or alkalis. The non-prolamin proteins (albumins and globulins) of wheat endosperm represent 20-25% of total grain proteins, the gliadins and glutenins constitute about 75-80%. Gluten is composed by interaction of the gliadins and glutenins. The main impact of the celiac disease is assigned to proteins member of the prolamin family. The epitopes responsible for allergenicity can be found both in the wheat prolamins and non-prolamin proteins. The quality and the composition of gluten proteins are very important from technological sight, determining the flour final utilization. So it is impossible to eliminate all of the toxic gluten protein fractions, because simultaneously the quality of bread making would be degraded. In case of non gluten proteins, among sufficient number of examined genotypes particular non-allergic gene variants could be found.

Previous research studies proved that, in spite of close genetic relationship among cultivars of wheat (*T. aestivum*) and spelt (*T. spelta*), spelt wheat resulted in better tolerance if products produced from its flour were consumed by people

suffering from wheat allergy. Differences mainly in water soluble proteins affected binding properties of IgE immunoglobulin in some European spelt cultivars. Similarly, products made from an Australian spelt genotype (GWF) possessing some mutations in a wheat allergy related expansin gene proved to be suitable to consume by patients.

Based on these problems and possibilities the aim of our project was to develop new strategies for breeding selection of less or non-immune reactive wheat species. A range of experiments were set up, which aim was improving the appropriate identification and detection of IgE mediated immunogenic proteins and sequences in bread wheat and spelta genotypes. The protein composition of the examined European and Australian spelt cultivars was characterized by different separation methods such as chromatography: SE-HPLC, RP-HPLC, Lab-on-a-chip, and one dimensional SDS- and A-PAGE and two dimensional gelelectrophoresis. 4 different protein fraction (albumin, globulin, gliadin, glutenin) of the spelt varieties were characterized. The result of this protein profile analysis showed significant differences in both the prolamin and non prolamin profiles of wheat and spelt. The GWF Australian spelt genotype, which has less immune reactivity as indicated by previous clinical experiment, showed unique seed protein composition compared to other examined spelt genotypes. The SDS gel profile showed different  $\omega$ -gliadin composition, some certain low molecular weight glutenins and  $\gamma$ -gliadins were absent.

Another promising way for finding out the allergen potential of cereal varieties is the application of molecular marker based techniques. Molecular markers are identifiable DNA sequences, indicating the presence of certain allergenic/toxic proteins. PCR-based experiments were performed to characterise polymorphisms of *T. aestivum* and *T. spelta* genotypes and to highlight differences between species. Based on a publicly available research articles and databases IgE-binding sequences were selected (thioredoxin, expansin,  $\alpha/\beta$ -gliadin, beta-purothionin, alpha-amylase inhibitor, different enzymes). A range of spelt and closely related species was screened using a set of identified allergen specific primers. Using these markers a multiplex PCR method was developed and used in marker assisted selection (MAS) in breeding programs in order to breed genotypes suitable for special needs.

# PROTEOMIC ANALYSIS OF ALLERGENIC PROTEINS IN WHEAT

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Nowadays large study on plant proteins, proteomics, is one of the most dynamically developing field of scientific research. Proteomic as a discipline allows for comprehensive exploration of plant tissues and also providing a wide range of information about the molecule under investigation. Wheat (*Triticum aestivum* L.) is an important cereal grain for export and domestic consumption in many countries throughout the world. The main use of wheat grain is the production of flour which, depending on the specific type of wheat, is used in many baked goods (Tatham *et al.*, 2008).

Wheat proteins can be divided into two broad groups on the basis of their biological functions. Albumins and globulins are biologically active enzymes associated with nutritional quality of baked goods. Gluten proteins (gliadins and glutenins) are biologically inactive storage proteins making up about 80% of the total (Payne *et al.*, 1985). The quality of bread and the other baked goods depends mainly on wheat's attributes like special storage proteins, gliadins and glutenins. However, these proteins are also connected with food and respiratory allergies (Baker's asthma) and intolerances (Coeliac disease) (Ciclitira *et al.*, 2003)

The aim of our work is to compare four genotypes of bread wheat (*Triticum aestivum* L.) based on the protein fractions using two-dimensional gel electrophoresis (2DE). This technique for quantitative proteome analysis combines protein separation by high-resolution (isoelectric focusing/SDS-PAGE) two-dimensional gel electrophoresis (2DE) with mass spectrometry for identification of proteins spots. One of the most important method within two-dimensional gel electrophoresis is protein purification. The most important in terms of defining the precise protein is the correct extraction of the total mixture of proteins. Our goal is to optimize the best method for the extraction of protein fractions of wheat and get the best proteomic gels. Since each fraction cereal proteins are characterized by solubility in different solvents, it is necessary to create the best protocols for their perfect finish to a solution and then isolate the protein from the mixture. Albumins were extracted from 200 mg of a milled grain sample that was mixed continuously with 1ml of buffer containing 25 mM sodium phosphate (pH 7.5) for 60 min at 4 °C. Gliadin proteins were extracted from 200 mg a milled grain sample using 1ml 50% (v/v) aqueous iso-propanol mixing at 1000 rpm for 30 min at room temperature. After mixing the both fraction of wheat were centrifuged at 5800 rpm for 15 min at room temperature. After centrifugation, supernatants were precipitation by 0.1M ammonium acetate in methanol and incubated at -20 °C over night. On the second day pellet of proteins were washed twice with 0.1 M ammonium acetate

in methanol, twice with 80% (v/v) ice-cold acetone and finally with cold 70% (v/v) ethanol. Samples (50 µg protein from each fraction) were diluted in aliquot volume of IEF buffer (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton X-100, 50 mM dithiothreitol) and added 1,25 µl ampholyte and loaded into pH 5-8, 7 cm immobilized pH gradient strips (ReadyStrip™ IPG Strips BioRad) for the isoelectric focusing. For second dimension IPG strips were incubated in SDS equilibration buffer (1.5 M Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol, 5% (w/v) SDS for 15 min with 2% (w/v) dithiothreitol followed) followed by a second equilibration step of 15 min with the equilibration buffer containing 2.5% (w/v) iodoacetamide. The equilibrated strips were loaded on the top of 30% polyacrylamide gel. After the completion of 2DE gels were stained for 16 hour using Coomassie Brilliant Blue G-250 at room temperature.

The presented experiments comparing different genotypes of wheat are only the part of big study about wheat proteins. The creation of two-dimensional protein gels of wheat grain will be followed by the analysis of individual protein spots excised and subjected to identification of the mass spectrometry. Data will be further processed by bioinformatics methods for the identification of proteins. The data collected will give us detailed information and comprehensive view of wheat protein.

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# THE GENES OF AN ORGAN DEVELOPMENT REGULATING TRANSCRIPTION FACTOR FAMILY IN *BRACHYPODIUM DISTACHYON*

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The impressive developmental capacity and morphological plasticity of plants are among the most important tokens of their adaptation to the continuously changing environmental conditions. During the accommodation process plants can modify their architecture in response to environmental stresses thanks to the dynamic regulation of meristematic cell activity. The molecular mechanisms regulating the boundaries between meristems and already differentiated organs are key components of this regulation process, and special cells at these boundaries take a crucial part in it. In accordance with their special function these cells express unique sets of genes, such as *CUC* (*CUP SHAPED COTYLEDON*), *NAC* (*NO APICAL MERISTEMS*) and *LOB* (*LATERAL ORGAN BOUNDARIES DOMAIN*) genes to form boundaries. The regulation of the meristematic activity through these genes is especially important for a proper and proportional organ development, including the formation of the whole root system (Rast and Rüdiger, 2008).

Since the formation of efficient root architecture is essential for plants to survive under limited water supply, the main subject of our research was the better understanding the effects of the above mentioned molecular mechanisms on the root developmental processes via physiological and molecular biological tools. For this purpose we used purple false brome (*Brachypodium distachyon*) as a model in our experiments. Thanks to the simple growth requirements, short life cycle, fully sequenced and relatively small genome and owing to the close phylogenetic relationship to agronomical important cereals (eg. wheat, barley, rye), this annual grass has become an accepted and widely used model plant for monocots in the last 10 years (Vogel et al, 2010, Darper et al, 2001). *Brachypodium* is particularly useful in researches investigating monocot specific developmental processes, where *Arabidopsis thaliana* would not be appropriate model being a dicot plant.

At first 31 various *Brachypodium* inbred lines were tested partially in rhizotrons and in pots in terms of their different responses to water limitation. On the basis of main component analysis it can be established that certain genotypes reply differently to drought stress in terms of general alterations in growth parameters and in root architecture. It was also revealed that deep-growing primary and nodal roots are essential for successful adaptation.

Among the candidates of root architecture influencing genes in this research we studied the LATERAL ORGAN BOUNDARIES-domain containing genes (*LBDs*) of *Brachypodium*. This gene family was first described in *Arabidopsis thaliana* as a plant specific transcription factor family. Their name refers to the observation that the spatial expression of the founding member of the family restricts typically to the boundaries between lateral organs (Shuai et al, 2002). In *Arabidopsis thaliana* 42 LOB-domain protein coding genes can be found. Their common feature is the presence of a conserved, 100 amino acid long LOB-domain structure which consist of a cysteine-rich CX<sub>2</sub>CX<sub>6</sub>CX<sub>3</sub>C motif suitable for DNA binding, a Gly-Ala-Ser box and a leucine-zipper like motif that allows protein-protein interactions (Matsumura et al, 2009). Although their exact functions are mainly unknown, on the basis of *Arabidopsis* experiments it has been demonstrated that *LBD* genes are involved in all aspects of plant development from embryogenesis to seed production owing to their crucial role in the lateral organ separation (Majer and Hochholdinger, 2011). In spite of the fact that their homologues can be found in almost all green organisms studied so far from more complex seaweeds to higher plants, aside from some exceptions we know hardly anything about their roles in monocots (Bortiri et al, 2006; Yoshiaki et al, 2005; Li et al, 2008). Therefore we aim to get to know in detail the processes controlled by *LBD* genes in *Brachypodium distachyon*, as a model system, with a major focus to root development.

We could identify 24 LOB-domain protein coding genes in the genome of *Brachypodium* which can be divided into two major classes and some minor subclasses on the basis of their sequence homology. For the identification of root specific ones we analyzed the relative expression of *LBD* genes via quantitative real-time RT-PCR in 37 different organs and various parts of the plants from root tip as far as to shoot apex, both in vegetative and generative organs. According to the expression pattern characterization we found that certain *LBD* genes have distinct organ specificity: some of them are definitely active in flowers, in various parts of the floret and in developing seeds, while some others showed high activity in green plant parts, and of course there were some *LBD* genes which can be described as root specific ones. Generally speaking, aside from few exceptions the closely related *LBD* genes have similar expression pattern. In addition to that our analysis goes far beyond the similar literature dates in terms of its detailed resolution, we got several expression patterns which have a good correlation to the transcriptional activity of their homologues that can be found in other plants (eg. *Arabidopsis*, rice). This suggests an evolutionary conserved function of *LBD* genes.

So far we selected two members from the 24 *LBD* genes for future analysis, namely the *LBD15* and *LBD13*, for two reasons: at first, they could be associated presumably with cell cycle regulation on the basis of our unpublished experiments related to their homologues in *Medicago truncatula*. For second, in despite of their very close phylogenetic relationship they show a significantly different expression profile from each other (namely *LBD13* showed a very sharp root tip specific expression, while the activity of *LBD15* is less organ-specific, and its relative transcript level was especially high in generative organs). The promoter analysis

confirmed the root tip specificity of *LBD13*, apart from the fact that promoter-GFP-reporter gene construction revealed activity also in the anthers; and the preliminary analysis of *LBD15* overexpressing *Brachypodium* seedlings suggests that LBD15 may have effect on germination, spike development, shoot and root growth.

## ACKNOWLEDGMENT

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# THE EFFECTS OF WHEAT GLUTENIN SUBUNITS ON THE FUNCTIONAL PROPERTIES OF RICE DOUGH

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Prolamins are storage proteins expressed in endosperm to conserve energy and nutrients for the germinating seed. The amount and distribution of prolamin proteins in wheat (*Triticum aestivum*) cultivars determine the dough mixing and bread making parameters of the flour. Prolamins are traditionally classified into monomer gliadins and polymer glutenins stabilised by inter-chain disulphide bonds. Gliadins are responsible for the extensibility, while glutenins influence the strength and the elasticity of the dough. Glutenin subunit proteins can be classified into the low molecular weight (LMW) and the high molecular weight (HMW) glutenin subunits. The glutenin genes are encoded on the 1A, 1B and 1D chromosomes, the LMW glutenin subunit genes are encoded at the Glu-3 loci on the short arm, while the HMW glutenin subunit genes at the Glu-1 loci on the long arm. The HMW locus contains two tightly linked genes, a larger and a shorter one, encoding the x- and the y-type HMW GS, respectively. Due to the variability of glutenin proteins in wheat flour it is hard to study the special properties of the unique proteins and their effects on bread making quality. Using rice (*Oryza sativa*) as a model system can solve this problem as the storage protein profile of rice is very different from wheat. Rice endosperm has a low amount of prolamin and the rice polymer proteins have globulin type subunits. Despite the differences in the protein compositions between the two cereals, it is still possible to make wheat bread-like product from rice flour with additives. These products are suitable for people suffering by allergic to certain wheat flour components.

Two methods can be used to study wheat glutens in rice model system. In the *in vitro* dough reconstruction studies purified proteins are added and incorporated into the rice flour to study their effect on the functional properties of the dough. The polymer structure of the dough can be broken by partial reduction and a new polymer matrix can be formed after addition of the protein of interest by re-oxidising the dough. In this experiment heterologously expressed HMW GS analogue proteins (ANG) – designed after a C-hordein gene, which does not contain any cysteine amino acid – were incorporated into rice flour. Three analogue proteins containing different numbers of cysteine residues on the N- and C-termini were studied. Analogue proteins with an odd number of cysteine residues (e.g. 1N and 2NIC) have a terminating effect on the polymer; however, proteins with an even number of cysteine residues (e.g. 2N2C) may enlarge the polymer, hence both can affect the dough mixing parameters.

In *in vivo* studies transgenic rice lines expressing glutenin subunits can be developed and the flour from transgenic seeds can be studied. In this work two different transgenic rice lines were generated: one expressing the HMW-GS 1Dx5 glutenin and one expressing both 1Dx5 and 1Dy10 proteins.

Two transformation cassette were established containing the sequences of *Glul-Dx5* promoter::*Glul-Dx5* gene::Nos terminator with the *hpt* selectable marker gene and the *Glul-Dx5* promoter::*Glul-Dy10* gene::Nos terminator with the *bar* selectable marker gene, respectively. Biolistic method was used for rice calli transformation. The selected transgenic plants were regenerated; the presence and expression of the transgenes were confirmed by PCR reaction and Western-blot analysis.

Three type of ANG protein were incorporated into the rice base flour. 2 mg/ml DTT solution was used for the partial reduction of the polymer and 5 mg/ml KIO<sub>3</sub> solution for re-oxidation, adding 10 mg analogue protein to 3.99 g rice flour.

The changes in rheological properties were observed by a prototype of z-arm mixer in case of both the incorporation and the transgenic studies. The effect of the recombinant proteins on polymer protein distribution was studied by size-exclusion liquid chromatography (SE-HPLC), one of the most common methods to investigate the polymer structure of dough. Results give important information about the distribution of protein fractions with different polymer state and the rate of soluble and insoluble polymer protein fraction (UPP% – unextractable polymer protein)

The incorporation of 1N and 2N1C ANG-proteins cause the dough to weaken due to their polymer-terminating effect. Significant differences were observed from the control flours, the resistance breakdown increased, while peak resistance and the dough stability decreased. The changes in the polymer structure of the dough were analyzed by SE-HPLC. Lower level of polymer protein and the decreased UPP% were measured in the dough containing 1N proteins compare to control doughs. These effects were not observed in case of the incorporated 2N1C proteins. Unlike the terminating ANG proteins, the 2N2C proteins provided stronger and more stable dough indicated by the increased value of the maximum resistance and the stability. The analysis of the polymer proteins in the dough resulted higher value in UPP% and unextractable polymer fraction. Similar results were observed in previous studies using wheat flour as a base-flour.

Transgenic rice lines expressing the wheat *Glu-1Dx5* gene were established. The presence of the transgene was confirmed by PCR. To monitor the transcription and the protein expression pattern of the transgene RT-PCR and Western-blot analysis were carried out. The expressed Dx5 protein improved the rheological properties. The peak resistance and stability was higher in the case of transgenic flour compared to non-transgenic flour, while resistance breakdown was decreased. By SE-HPLC analysis higher polymer content and UPP% were measured.

Rice lines expressing Dx5 protein were transformed with the transformation cassette containing *Glu-1Dy10* gene, and the integration of the two genes were detected by PCR. The expression of the transgenes was confirmed both in RNS and protein level. The alteration of the polymer structure in transgenic endosperms was

monitored and an increased amount of unextractable polymer protein fraction was observed.

*In vivo* and *in vitro* studies indicated that either incorporated or expressed wheat glutenin proteins have significant effect on rheological properties and polymer formation of rice dough. Incorporation of disulfide bond-forming wheat glutenins into prolamin-poor rice flour as well as the expression of glutenin proteins in transgenic rice endosperm improved its dough making parameters. Investigation the effect of distinct glutenin subunit proteins on the rice flour including the interactions with endogen rice proteins, could result a transgenic rice line expressing several wheat glutenin proteins with proper baking properties but not causing allergic symptoms in people suffering by globulin type wheat allergy.

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# “GLOBALIZATION” IN THE SEXUAL REPRODUCTION OF CEREALS

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Ultrawide hybridization (UWH), i.e. non-GM-based trait transfer by sexual crossing between distantly related species or genera, has long been applied to introduce novel genes into crop plants. Major hurdles prohibiting more widespread and routine use of UWH have been the low frequency and decreased viability of hybrid embryos, which make embryo rescue and nursing plants by tissue culture compulsory steps in the process. Beyond its agronomical importance UWH also offers a fascinating model to study early phases of reproductive development. Here we asked whether low success rates of UWH in cereals are caused by a low frequency of fertilization (including egg cell activation and pollen tube formation) and/or by incomplete or blocked endosperm development.

Using an optimized wheat (♀) × barley (♂) hybridization system (Figure 1) and via a comprehensive microscopical study we found that pollen tube formation (Figure 2) and fertilization can successfully be accomplished. Thus, early steps of sexual reproduction, including egg cell activation, are not inhibited in these intergeneric hybrids. Endosperm development, however, was essentially blocked at the onset of the cellularization, which can cause early embryo abortion due to the lack of stored nutrients. Via suppressing this endosperm block we were able to produce normal, endosperm-containing wheat-barley hybrids. As a result, harvested hybrid seeds germinated readily without the aid of tissue culture. The majority (ca. 80%) of hybrid plants contained a partial or the full, seven-chromosome complement of barley.

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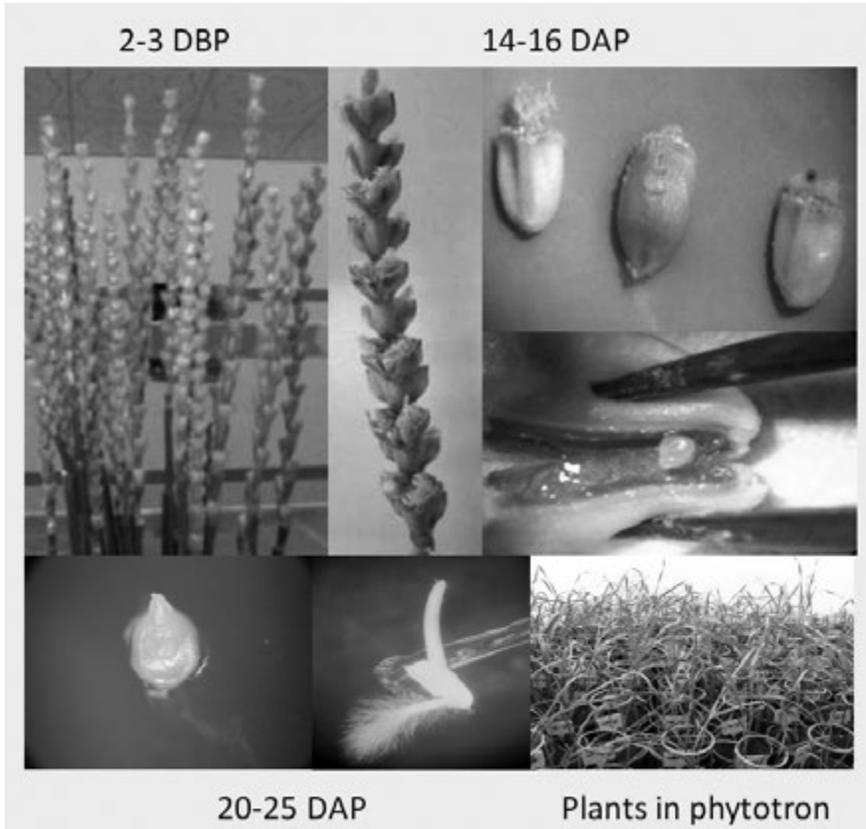


Figure 1. Step by step presentation of the wheat x barley hybridization procedure: emasculated and pollinated spikes, immature pseudoseeds, embryo rescue, *in vitro* germination and plant regeneration, nursery in pots. DBP, days before pollination; DAP, days after pollination.

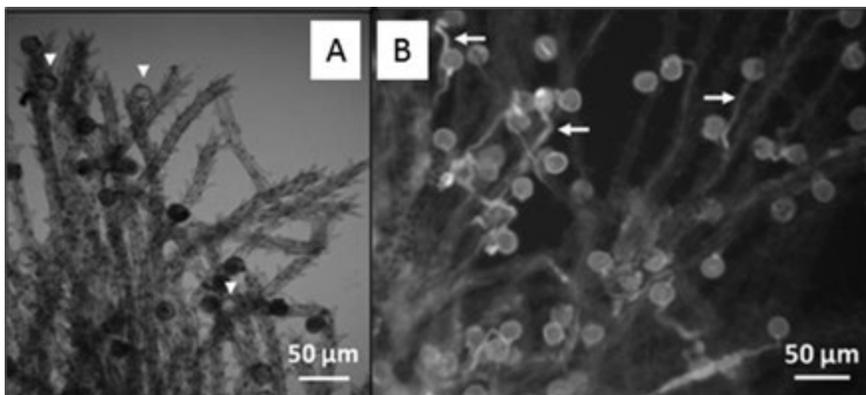


Figure 2. Microscopic view of the barley pollination process on wheat pistils. (A) Viable barley pollen grains (arrowheads) 1 hour after pollination as revealed via aniline blue staining. (B) Subsequent pollen tube formation (arrows) detected using trypan blue stain.

# IDENTIFICATION OF MITOGEN-ACTIVATED PROTEIN KINASE SUBSTRATES IN ARABIDOPSIS PROTOPLAST TRANSIENT EXPRESSION SYSTEM

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Plant growth is highly dependent on changing environmental conditions, due to their sessile lifestyle. Therefore understanding the mechanisms by which plants regulate adaptation to environmental factors is of paramount importance. Inside the cell, environmental stimuli are mediated by signal transduction pathways. Protein kinases are important components of signalling pathways, they can change the activity, stability or localisation of their substrate proteins by phosphorylation of certain residues. Mitogen-activated protein kinase (MAPK) phosphorylation cascades are well-conserved signalling modules in all eukaryotes, which play key roles in stress signal transduction, regulation of cell division and cell growth.

The human kinome takes up about 2% of the genome, the *Chlamydomonas* kinome is slightly more than 2%, whereas about 4% of the *Arabidopsis* genome encodes kinases, probably because sessile lifestyle demands sophisticated signalling. This difference indicates a dramatic expansion of kinase regulatory networks during land plant evolution, implying the evolution of intricate signalling mechanisms necessary to survive terrestrial stresses (drought, salinity, temperature shifts, UV, etc.). Accordingly, flowering plants outnumber mammalian systems in terms of MAPK signalling genes, which have emerged by consecutive duplications of the relatively simple ancestral plant MAPK signalling set.

In plants, MAPK pathways have been mainly shown to play important roles in stress signalling, but they are also involved in regulating plant developmental processes. Although there are about twice as many kinases in plants as in mammals, our knowledge of kinase substrates in plants is scarce. For example, there are only about ten known *in vivo* substrates in the model plant *Arabidopsis*. In comparison, ERK1/2, the human MAPKs that are most similar to plant-type MAPKs, have over 100 different substrates. Because knowledge of substrates is key to functional understanding of MAPK pathways, the aim of our research is to identify novel MAPK substrates in plants.

To this end, we have developed an efficient experimental system, in which substrate candidates are transiently expressed in protoplasts with or without co-expression of activated MAP kinases. Changes in phosphorylation status of the expressed proteins are detected based on the separation of differently migrating

phosphorylated and non-phosphorylated protein isoforms from protein extracts. We analyse the samples using a novel technique based on capillary isoelectric focusing coupled with nanofluidic immunoassay. In this system, protein isoforms of varying isoelectric points are separated by isoelectric focusing in a capillary, then immobilized, and probed with antibodies. This is an extremely sensitive system, suitable for quantitative measurements, whereby subtle changes in isoform distribution can be detected.

Capillary isoelectric focusing coupled with nanofluidic immunoassay is a novel, state-of-the-art method, which – to the best of our knowledge – has not been used in plant research. Furthermore, in our approach the samples are produced in transfected protoplasts, which enable rapid and flexible experimental arrangement. We believe that this efficient experimental approach to identify kinase substrates in plant cells has a great potential to contribute to our understanding of signalling pathways in plants.

# REGULATION OF PROTECTIVE PROLINE SYNTHESIS IN *ARABIDOPSIS THALIANA*

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Environmental stresses impact on all aspects of plant architecture and represent a serious challenge for developing sustainable agriculture at a time of significant growth in the global population. To cope with these stresses, plants have evolved a wide spectrum of molecular programs to sense change rapidly and adapt accordingly. Understanding, and – if it is possible – improving these reprogramming events under constantly changing environmental conditions has been a subject of great interest (Ahuja *et al.*, 2010).

Plants have evolved diverse strategies of acclimatization and avoidance to cope with adverse environmental conditions. Proline, as free amino acid is common among stress-induced metabolites and has been shown to accumulate during different environmental stresses including drought, salinity, and oxidative stress; moreover proline level responses to certain biotic stresses (Szabados, 2010). Several protective functions were attributed to proline, such as scavenging ROS, acting as osmoprotectant and maintenance of redox equilibrium. Interestingly, proline has been shown to protect plants against singlet oxygen and free-radical induced damages. Due to its action as singlet-oxygen quencher and scavenger of OH• radicals, proline is able to stabilize proteins, DNA and membrans (Matysik *et al.* 2002).

The *in vitro* use of reactive carbonyls, like methylglyoxal or glycolaldehyde is a straightforward method to imitate the ROS mediated *in vivo* damages.

To confirm this theory, we examined the protective effects of proline on glycolaldehyde treated lactate-dehydrogenase. Protein oxidation assays for detection of protein C=O groups involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product. This can be detected by polyclonal  $\alpha$ -DNP antibody after one-dimensional electrophoresis followed by western blot (Job *et al.* 2005). The *in vitro* enzyme activity was measured photometrically at 340 nm following the consumption of NADH cofactor (Luschak *et al.* 1998).

We can conclude that proline can not directly protect this enzyme from oxidation in *in vitro* assays. Several assays demonstrated that at 30 mM proline concentration protein oxidation and crosslinking is the most pronounced.

In enzyme activity assays results showd that (1) at low concentration of glycolaldehyde shows that high concentration of proline can protect the LDH enzyme against the carbonyl caused damage. (2) The enzyme retains more than 50 % of its initial activity in the presence of high proline despite the much (5-fold) more inhibitor concentration (3) preincubation of proline and glycolaldehyde

significantly decreased the inhibitory action of the reactive carbonyl compound at 100mM proline concentration. This may show the direct reaction between reactive carbonyl compounds and proline.

The *in vivo* experiments were carried on *Arabidopsis thaliana* (Columbia ecotype). In A. th. the synthesis of prolin is performed by two enzymes. The P5CS2 localized in the cytosol and acts as a housekeeping enzyme. The stress inducible P5CS1 accumulates and in the chloroplast and producing proline during stress conditions. The centre of our interest is the *P5CS1*.

In earlier *in silico* analyses showed that in the *P5CS1* promoter, transcription factor binding sites from G-Box and MYB families can be found.

The yeast one-hybrid system is a powerful method to identify heterologous transcription factors that can interact with a specific regulatory DNA sequence of interest. Detection is based on the interaction of a transcription factor (prey) with a bait DNA sequence upstream of a reporter gene. In the course of the experiments on this gene we focused on its methylation pattern too, because these posttranscriptional modifications can cause significant alterations in gene expression. In the promoter fragment of *P5CS1* next to the potential transcriptional factor binding sites, a theoretical small RNA binding site and a potential methylation site were identified.

By the McrBc digestion of isolated plant DNA (which enzyme cleaves at methylated cytosines) followed by PCR, we can make the methylation profile of the promoter and the gene body. Therethrough we can conclude that the abovementioned DNA fragment is the mostly methylated region of the promoter, maybe it has an important role in the regulation of gene expression. We can alternate the methylation pattern by treating the plants with 5-azacitidin *in vivo*. This chemical acts by inhibiting enzymes that methylate cytosine residues in eukaryotic DNA and can activate genes by changing their methylation status. This way we can have a more focused point on the relation between the methylation set(status) of the gene and its expression level.

These results suggests that the methylation pattern of *Arabidopsis thaliana P5CS1* shows a dynamic phenomenon upon development and stress response.

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# FUNCTIONAL ANALYSIS OF CANDIDATE VIRULENCE GENES OF PHYTOPATHOGEN FUNGUS *VERTICILLIUM ALBO-ATRUM* PREDICTED BY FUNGAL COMPARATIVE GENOMICS AND PROTEOMIC ANALYSIS

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*Verticillium albo-atrum* is a soil-borne plant pathogen responsible for *Verticillium* wilt diseases. Together with *Verticillium dahliae*, it belongs to the genus *Verticillium*, which causes billions of dollars in annual crop losses worldwide. Collectively they affect over 200 hosts, including many economically important crops, such as oilseed rape, cauliflower, potato, cotton, tomato and lettuce (Fradin and Thomma, 2006; Klosterman, 2009). *Verticillium* wilt is also a very important disease in Slovenia, infecting hop (*Humulus lupulus L.*). In 2013, Slovenia held fourth place in world production of hop. Hops were grown on 1166 ha, of which 40,6 ha ( $\approx 3.5\%$ ) was infected with the lethal pathotype of *V. albo-atrum*. *Verticillium* wilt has been detected on 204 ha of hop fields since 1997 (Radišek, 2013), which makes it a severe economic problem for hop production in Slovenia. The management of hop wilt could be greatly improved by better understanding the mechanisms of pathogenesis and interaction between plant and pathogens on a molecular level. Here, we present our work on the search for the *V. albo-atrum* (Vaa) virulence factors.

## IDENTIFICATION OF CANDIDATE VIRULENCE GENES

### *In silico*

The Slovenian Vaa genome of the lethal strain (PV1), isolate T2, was sequenced as reference genome with high coverage and assembled with the aid of an optical map. Ten pseudomolecules were obtained in a total length of 35.64 Mb, while an additional 2.91 Mb of contigs remained unplaced. The gene models were constructed using several gene prediction and comparison tools (GeneMark, Augustus, Blast, Exonerate) supported by RNA-seq analysis (Cufflinks) and combined by EvidenceModeler, revealing 9,858 gene models in total, which were annotated with blastX comparisons using the blast2go annotation tool. Five other Vaa strains from Slovenia, Germany and England were sequenced using a shot-gun approach. We focused on gene models residing in the lethal specific region of our PV1 Vaa isolates. Selected candidate nucleotide sequences from the lethal specific genomic region were analysed for

effector characteristic nucleotide features using various algorithms (de Jonge, 2012, Saunders *et al.* 2012). The *in silico* analysis gave a list of candidate effectors and some of them were tested for their role in virulence by deletion mutation analysis.

### *In planta*

Xylem sap proteins extracted from a susceptible and a resistant hop cultivar infected with the fungus Vaa were analyzed by 2D electrophoresis. We were able to resolve 200-250 protein spots on 2D gels in a pH range of 3-10 and molecular weight range of 10-100 kDa, most of them being plant proteins. No substantial differences were found between infected and control xylem sap of the resistant cultivar, while highly abundant fungal proteins were detected in the xylem sap of infected susceptible plants. Three different fungal secretory proteins were identified by mass spectrometry: 14 kDa hypothetical protein and CWDEs alpha-N-arabinofuranosidase and versatile peroxidase VPL1.

## A PROTOCOL FOR GENERATING AND TESTING KNOCK-OUT GENES OF *V. ALBO-ATRUM* AGGRESSIVE STRAIN

Our protocol for generating knock-outs of the fungal pathogen *V. albo-atrum* comprises two methods: the creation of knock-out plasmids by the USER Friendly cloning technique and transformation of the fungal pathogen by *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Knight *et al.*, 2009). Knock-out strains of *V. albo-atrum* were made by site directed modifications of the pathogen genome by means of homologous recombination and achieved by introducing a DNA fragment containing two homologous recombination sequences flanking a selection marker. pRF-HU2 plasmid, containing a hygromycin resistance gene, was used for user friendly cloning of knock-out plasmids (Frandsen *et al.*, 2008). Two PCR amplicons, containing homologous recombination sequences flanking a deletion gene, were inserted into the vector and the entire user treated reaction mixture was used to transform chemically competent *E. coli* cells by heat shock. Isolated knock-out plasmids from *E. coli* were transformed into electro-competent *A. tumefaciens* by electroporation. *V. albo-atrum* knock-out transformants were generated by ATMT. *V. albo-atrum* knock-out transformants were verified by PCR testing, which confirmed that deletion of the target gene had been successful.

## TESTING KNOCK-OUT TRANSFORMANT VIRULENCE ON HOP (PATHOGENICITY ASSAY) AND GENOMIC COMPLEMENTATION OF THE KNOCK-OUT GENE

The hop plants were used in the pathogenicity assay to evaluate the effect of knock-out of the target gene using the root dipping method for infection (Radišek *et al.*, 2003). The infected and control plants were grown in growth chambers under controlled conditions. Foliar wilt symptoms were assessed at sampling

time points, which were determined at 10, 20 and 30 dpi. Plants infected with knock-outs were compared to control plants (wild type infected plants and mock infected plants) to assess the mutation effect on the virulence capacity of Vaa. A 0-5 scale and a disease severity index (DSI) was calculated for hop wilt symptom assessment (Radišek *et al.*, 2003). The presence of the pathogen in inoculated plants was confirmed by mycological re-isolation of the pathogen and, if necessary, by qRT-PCR. If knock-out transformants had shown reduced virulence on infected plants, genomic complementation of the knock-out gene was performed. By genomic complementation, the deleted targeted gene is reinserted in the knock-out transformants. The effect of complementation was tested by pathogenicity assay on host plants. This is a very important test, which is ultimately needed to confirm the tested gene role in virulence.

So far, we have identified one *in planta* gene (14 kDa hypothetical protein) and four *in silico* genes which knock-outs showed reduced virulence.

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# TRICHOMES TYPES AND THEIR DENSITY IN PARENTAL SPECIES *SOLANUM TUBEROSUM* AND *S. CHACOENSE* AND THEIR DERIVED SOMATIC HYBRIDS

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Trichomes represent an insufficiently explored domain, but with an extraordinary potential, with a whole lot of valuable practical applications. Trichomes are metabolic factories involved in the interrelationship between plants and herbivores.

*Solanum chacoense* is a wild species of potato, well known for its resistance to Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*). It reveals also, sexual compatibility with *Solanum tuberosum*, the commercial species, which is situated currently, on the third position in the international top of the most cultivated agricultural plants. One of the natural resistance mechanisms of the plant is represented by trichomes besides resistance conferred by specific leptines glycoalkaloids. The trichomes are extensions of the epidermis, also known as hairs. There are several types of them, depending on their length, morphology, cells number or even substances that they are synthesizing or secreting. Different types as glandular or non-glandular, uni- or multicellular hairs as well as long or short trichomes were described in the literature, and morphological types were classified. For the interrelationship with herbivores glandular trichomes seems to be the most important but there is no further clue of what kind of reaction but mechanical they induce, at least in potato crop and related *Solanum* species.

The purpose of this study was to identify differences regarding morphology or density, utilizing comparative analysis between plants representing parents and somatic hybrids between potato + *S. chacoense*.

Potato cultivars 'Delikat' and 'Désirée' and the accession of *S. chacoense* PI 458310, high leptine producer (HL) (provided by NPGS Sturgeon Bay, USA) have been used for mesophyll protoplast isolation, electrofusion and somatic hybrid plant regeneration (data not shown). Part of the experiments involved transgenic *S. chacoense* deficient in mismatch repair system (MMR) by the transfer of antisense or dominant complementar negative mutant of the gene *Atmsh2* (*msh2* gene with specific sequence of *Arabidopsis thaliana*). Trichomes analysis was technically supported by light and fluorescent microscopy (Olympus BX60 with video camera XC50 and LabSens software). For density determination the same leaf surface area from similarly developed plants grown *ex vitro* in a phytothron were used. Stereomicroscope was used to visualize the leaf abaxial hairs and digital pictures were recorded (Olympus digital camera Camedia C-5060) by using the same magnification of stereomicroscope and computer assisted image assessment. The density was evaluated on veins and interveins

leaf surface, in at least five leaves and three different randomly chosen leaf areas of each genotype and then total glandular or non-glandular hairs were calculated. Data were statistically analyzed for standard deviation and significance (t test).

The principal types of glandular or non-glandular trichomes were identified based on the ones reported in the literature. Details of morphology and possible fluorescent compounds were analyzed under epifluorescence using different filter combinations. Dual observation with both light and fluorescence revealed new spatial details of the hairs in abaxial epidermal tissue microscopic preparations.

Studying the morphology of trichomes on the plants, among the eight types specifically described in *Solanum* genus, five of them were identified, namely: II, III, V, VI and VII. Across the board, there are two categories of hairs: glandular and non-glandular. Type II is a non-glandular, short hair, with a multicellular, globose base. Type III is also non-glandular, composed of 4-8 cells and a unicellular, wide base. Another non-glandular type is the V, which has a single cell and a short, voluminous construction. Types VI and VII, are both glandular. The first of them is short, voluminous, with a bicellular stem and the head composed of four cells. The second type describes very small hairs, with a termination made of 4-8 secretory cells. In contrast to the leaves, on the plant stems only non-glandular trichomes were detected, which are long and made up of a great number of cells.

Analyzed somatic hybrids tend to keep non-glandular hairs from the wild species *S. chacoense*. Regarding similitude with potato (*Solanum tuberosum* 'Delikat' or 'Désirée'), trichomes present on the hybrids leaves are different. Cultivated potato hairs do not present any fluorescence. Instead, the wild species and hybrids had this feature. The non-glandular trichomes in both wild species and hybrids reveal a yellow fluorescence under blue filter in vicinity of the cell wall; glandular trichomes have a faint green fluorescence under the same filter cube (seen as blue) in all cells, caused probably by the accumulation of flavonoids.

Also, double light observation emphasizes a lot of morphological details which can't be seen under optical microscope with direct light, like tridimensional structure and drops of secreted compounds.

The density of the hairs on leaf abaxial surface varies significantly, both for glandular and non-glandular hairs and between veins and non-vein areas. *S. chacoense* presents a low density of robust non-glandular trichomes but a high density of the glandular ones. In contrast potato cultivars are characterized by high density of non-glandular type III hairs. In the hybrids a lot of variation in density is to be seen.

In conclusion: (i) trichomes densities and types vary in the somatic hybrids analyzed, with some having very high and some low densities; there is variation also in the same hybrid. These data are going to be correlated with resistance to CPB and leptines biosynthesis; (ii) the wild species and the majority of the hybrids present a green fluorescence that might be caused by flavonoid biosynthesis in the glandular trichomes.

## ACKNOWLEDGEMENT

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# THE EVALUATION OF RESISTANCE TO COLORADO POTATO BEETLE AND DETECTION OF GLYCOALKALOID CONTENT BY USING FOURIER TRANSFORM INFRARED SPECTROSCOPY IN *SOLANUM TUBEROSUM* + *SOLANUM CHACOENSE* SOMATIC HYBRIDS

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The cultivated potato *Solanum tuberosum* ranks third in agricultural crops production on global scale, being the target of attacks by various insects. The Colorado potato beetle (CPB) has meanwhile become the biggest enemy of the cultivated potato worldwide. The CPB is very voracious and shows high adaptability to biotic and abiotic changes and multiple resistances to several insecticide classes (organophosphates, carbamates, organochlorines, arsenicals and pyrethroids). Insecticides are also expensive and associated with risks to human health and the environment.

An alternative method of controlling the growth of CPB population would be the use of resistant potato varieties. One of the most effective sources of host-resistance mechanisms to CPB is the natural resistance of the wild species *Solanum chacoense*. Resistance to CPB is associated with the expression of rare glycoalkaloids, the leptines which have anticholinesterase-type activity. Leptines are only expressed in aerial tissues and not in tubers, which is important to consider regarding the potential consumer toxicity.

The development of biotechnology made possible the use of electrofusion of protoplasts to bypass the hybridization barriers between *Solanum tuberosum* and *Solanum chacoense*. Therefore one is able to perform the introgression of multiple genes for durable resistance to CPB into cultivated potato.

The aim of this study was to follow the development and the viability of CPB larvae fed on somatic hybrid plants. Also, the total chemical composition of somatic hybrids was compared with parents (cultivated potato, *S. chacoense*) using FTIR spectroscopy.

A laboratory bioassay was used to study the feeding and growth of CPB larvae. After the larvae hatched from eggs their weight was measured and they were transferred on different varieties of potato leaves (SHs, BC1 and the two parents' leaves). From 15 to 25 larvae were separately monitored for each plant variety. Every

second day the larvae were fed with fresh leaves, their weights measured, life stages noted and the number of dead larvae recorded. The larvae were monitored until more than 50% of them fed on cultivated potato were developed into adults.

In the second part of our research the total chemical compound of somatic hybrids and parental lines were analyzed (*S. chacoense*, *S. tuberosum*) using Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy is a simple and a rapid technique, based on the measurement of a molecule excited by IR radiation at a specific wavelength range. In the current case the mid-IR region was used for analyses. When IR radiation passes through a sample, specific wavelengths are absorbed, causing the chemical bonds in the material to undergo vibrations such as stretching, contracting and bending. The spectral peaks are derived from the absorption of bond vibrational changes in the IR region.

In the experiment hybrid and parent plant leaves were grounded into a fine powder under liquid nitrogen, and 1 ml of 70% methanol was added, and mixed by sonication for 15 minutes, then centrifuged. 100  $\mu$ l of supernatant was used for analysis. Infrared profile was realized using the Shimadzu Prestige 2, Apodization: Happ-Genzel spectrophotometer. FTIR measurements were performed in absorbance mode; the profile was recorded in the wavelength range of 4000-500  $\text{cm}^{-1}$ . Spectra analyses were processed using IRsolution software and Origin 7 software.

It took around 25-32 days for larvae fed on leaves of different potato varieties to complete their development passing through four larval instars (L1-L4) and a pupa before emerging as an adult. The use of a laboratory bioassay revealed that the high leptine content SHs and BC1 lines have toxic effect and greatly influences the viability and development of the CPB larvae. The resistance of some hybrid plants was equal, or it approached the resistance degree of *S. chacoense*. This behavior seems to be dependent on the presence of leptines. These resistant hybrids represent an important step forward in combating the CBPs.

The results of the FTIR analyses shows that the spectrum of resistant hybrids are similar to the spectrum of *S. chacoense* and the susceptible hybrid's spectrum is alike *S. tuberosum*'s spectrum. The resistant hybrids have similar chemical compounds and volumes as *S. chacoense*. This observation is based on the sameness of their IR spectra.

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# BIOPHYSICAL CHARACTERISATION AND *IN VITRO* DIGESTIBILITY OF SUNFLOWER STORAGE PROTEINS

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Some storage proteins of sunflower seed are able to stabilise food emulsions, such as mayonnaise, due to their excellent emulsifying properties. Some of the storage proteins of sunflowers have been proved to elicit allergic reactions in sensitive individuals, causing real danger to the patients. Due to the wide use of sunflower seeds it establishes a global risk to consumers.

A shared feature of allergenic food proteins is the ability to keep their structural integrity during food processing, consumption and digestion. The heat treatment and emulsification occurring during these processes may alter some properties of the proteins and therefore their allergenicity might be modified as well. Better understanding of the gastro-intestinal processing, the digestion of protein emulsions is necessary for developing less allergenic food products. In order to get better understanding of certain processes of food preparation affecting the allergenicity of the studied proteins by modifying the resistance to proteolysis, we investigated how the structure of the proteins changes upon heat treatment.

Those proteins of sunflower which had been found potentially able to stabilise food emulsions earlier were investigated. The lipid transfer protein of sunflower (LTP) and the SFA8 protein belonging to the 2S albumins, and three mixed fractions (signed as A, B and C) comprised of mainly Alb1 and Alb2 were studied. The change of the secondary structure upon heat treatment was followed by far UV-circular dichroism spectroscopy. We found that only small changes occurred in the molecular structure of the 2S albumins (Alb1, Alb2 and SFA8) of sunflower on heat treatment. The structure of LTP was stable as well, it showed only minor changes upon heat treatment but after cooling it did not refold to its original conformation perfectly.

We searched for the transition temperature of the proteins by differential scanning calorimetry but both LTP and SFA8 were resistant to the applied heat treatment.

Surface dilatation measurements at air/water interface were carried out. Surface tension at oil/water interface was measured by pulsating drop method. The surface activity studies are also able to detect small changes in the proteins' structure. The surface activity measured at the oil/water interface was affected by the applied heat treatment of the five studied fractions of sunflower storage proteins. The heat treatment increased the surface activity largely in case of LTP. In case of the mixed fractions (A, B and C) and the SFA8 the surface activity was raised in a smaller

extent by heat treatment. Only the SFA8 exhibited excellent surface activity, the LTP and the three mixed fractions (A, B and C) showed poorer surface properties.

Emulsions were prepared of solutions of the five proteins and oil. The size of the particles of the emulsions was measured. LTP was able to stabilise emulsions to a modest extent, while the mixed fractions were not able at all. Only the SFA8 was able to form stable emulsions, its emulsions possessed outstanding properties, such as small drop size and long term stability.

Both LTP and SFA8 are able to elicit an allergic reaction in sensitive individuals. Alb1 and Alb2 possess such typical structure (the so called 'LTP-signature') which is representative to allergenic proteins, so presumably they might be allergenic as well. Therefore the resistance to proteolysis in an *in vitro* digestion model of LTP and SFA8, and three mixed fractions of Alb1 and Alb2 proteins (fractions A, B and C) was also investigated. The pattern and the timing of the proteolytic degradation of the proteins were monitored. Two phases of the physiological digestion were simulated: the gastric phase occurring in the stomach (1<sup>st</sup> phase) and the intestinal phase which takes place in the small intestine (2<sup>nd</sup> phase). The effect of the emulsification and the presence of liposomes of phosphatidylcholine (a lipid present *in vivo* in the gastro-intestinal tract) on the digestibility of the proteins were studied. We found during the *in vitro* digestion that all the proteins of sunflower we investigated in this study (LTP as well as the 2S albumins) are digested in the artificial gastro-intestinal model. LTP and the 2S albumins retain their structural integrity even after being cleaved by the proteases because the fragments are held together by disulphide bonds during the gastro-intestinal digestion. Presumably, the fragments are able to get to the small intestine and pass through its mucosa eliciting an allergic reaction. Phosphatidylcholine had a defensive effect both in gastric and intestinal phase of digestion on LTP as well as the 2S albumins of sunflower: the mixed Alb1 and Alb2 fractions and pure SFA8. Emulsification provided an increased protection for SFA8 against proteolysis. (Only SFA8 is able to stabilise emulsion to sufficient extent amongst the proteins studied here.) Best protection was achieved when phosphatidylcholine and emulsification were applied together on SFA8. It indicates that both the lipid-protein interactions and the emulsification can modify a protein's allergenicity.

As a conclusion, it is not enough to test only the pepsin digestion when assessing novel proteins allergenicity; the proteolysis of trypsin and chymotrypsin also has to be taken into consideration as well as the multiphase nature of the gastro-intestinal tract and the lipid-protein interactions. Although the four studied proteins, especially SFA8, possess good surface properties and could be used as stabilisers, their use in food emulsions should be thoroughly considered because of their allergenicity.

# PROTEOMIC ANALYSIS OF FLAX ADAPTATION IN CHERNOBYL AREA USING GEL-FREE AND GEL-BASED APPROACH

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On 26 April 1986, the accident at the Chernobyl Nuclear Power Plant (CNPP) released vast amounts of radionuclides into the environment that contaminated large parts of Europe. Majority of radionuclides decayed in a few years since the accident. However, long-living radionuclides such <sup>90</sup>Sr and <sup>137</sup>Cs are still present in the environment (Møller and Mousseau, 2006). Surprisingly, plants were able to adapt Chernobyl radio-contaminated environment. The aim of this study was to use gel-free and gel-based proteomic approaches in order to elucidate adaptive mechanisms used by plants in the Chernobyl area.

For this purpose, flax plants were grown in radio-contaminated and non-radioactive experimental fields. Radio-contaminated field was located about 5 km from CNPP, near village Chystogalivka. Non-radioactive field was established in Chernobyl town. Developing flax seeds were harvested 14, 21, 28 days after flowering and at mature stage.

Proteins were isolated using phenol based protocol according to Hurkman and Tanaka (1986).

For a gel-based proteomic approach we used protein two-dimensional electroforesis (2-DE) in combination with tandem mass spectrometry (MS/MS). The 2-DE gels were prepared in biological triplicate using 17 cm IPG strips with pH 5-8, stained with Coomassie Brilliant Blue, and analyses by ImageMaster 4.9 in order to detect and quantify 2-DE spots. The 2-DE spots that were presented in both data sets (from radioactive and non-radioactive fields), at least in two biological replicates, and three or more developmental stages were accepted to the analysis. In total 130 spots satisfied these criteria. Out of these, 75 2-DE spots were found differentially abundant between experimental fields. These spots were excised from 2-DE gels, digested by trypsin and analysed by MS/MS. The MS/MS spectra were processed by ProteinLynx Global Server against the protein database that contained linum sequences from UniProt, NCBI and Phytozome. Identified proteins were classified into functional groups according to Bevan *et al.*, 1998. The biggest group was Protein destination and storage containing 40 proteins following by 17 energy proteins. Eight proteins were involved in disease/defence, and 6 were metabolic proteins. We also found 3 proteins associated with cell growth/division. One protein was

identified as a transporter, 1 protein participated cell structure, and 1 protein had unclear classification.

In case of gel-free analysis, we used the same plant material. Proteins were firstly incubated in dithiotreitol (DTT) in order to reduce the disulfide bonds and in iodoacetamide (IAA), that bind covalently with the thiol group of cysteine so the protein cannot form disulfide bonds backwards. Before MS/MS analysis, proteins were cleaned with Agilent Peptide Cleanup C18 Spin Tubes and digested by trypsin. Resulted MS/MS spectra were processed against the same database as in gel-based proteomics. This approach resulted into the identity of 136 proteins that were classified according to Bevan *et al.* Most of proteins were associated with Protein storage and destination group (86 proteins) and 30 proteins were unclassified. Group Disease/defence contained 6 proteins, Cell growth 3 proteins, 3 proteins were associated with energy and 2 in metabolic reactions. Additionally, one protein was associated with signal transduction.

These data suggest that mobilization of seed storage proteins and increased amount of proteins associated with stress response are involved in adaptation mechanism against ionizing radiation.

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# RECENT ADVANCES IN STYRIAN OIL PUMPKIN BREEDING

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In Central Europe styrian oil pumpkin (*Cucurbita pepo* ssp. *pepo* var. *styriaca*) is gaining ever greater interest in agriculture due to an increasing demand for aromatic seed oil and for other uses. Having a rather limited genetic pool, a lot of effort has been put into germplasm enhancement in the last decades, resulting in the release of hybrids, but in terms of modern breeding techniques styrian oil pumpkin can be considered as a minor crop. To accelerate breeding the development and optimization of biotechnological approaches is needed in order to meet the genotype-specific requirements. Our research mainly focused on the development of doubled haploids for hybrid breeding and studies on the applicability of somaclonal variation.

Haploid induction and subsequent genome doubling offers a time-effective alternative to the traditional development of pure lines for hybrid breeding. As known from literature (Kurtar *et al.*, 2002, 2009; Kurtar and Balkaya, 2010) pseudofertilization with irradiated pollen seems to be the method of choice in *Cucurbita* sp. Recent developments on the restriction of gamma ray use for research purposes (FAO/IAEA, 2013) suggest alternative strategies, one of them being X-ray irradiation. We tested several factors known to affect haploid production such as genotype of female plants and pollen donor, irradiation dose and season. We found that all factors affected fruit set, embryo formation as well as haploid induction rate. The best parthenogenetic response among genotypes used as female plants was found in 'Turkey #2' (10.0%), 'Gleisdorfer Ölkürbis' (4.4%), and 'Naked Seed' (3.9%), whereas 'GL Opal' and 'White Acorn' were efficient as pollen donors. The ploidy level of 3830 putatively parthenogenetic embryos was determined using flow cytometry with the majority being diploid. Interestingly, a significant proportion was determined to be tetraploid and this was clearly correlated with increased radiation delivered to pollen grains. The observed haploid frequencies were relatively low and no spontaneous genome doubling could be confirmed by SSR marker analysis (Košmrlj *et al.*, 2013) therefore we focused on the application of higher irradiation doses (> 350 Gy). Based on the Brewbaker and Kwack (1963) protocol we optimized the method for styrian oil pumpkin pollen, which is known to have short longevity even under natural conditions (Nepi *et al.*, 2010). In order to maintain pollen germinability after prolonged irradiation, exposure of pollen to high air humidity during irradiation was tested and compared to irradiation at room humidity. It was found that it significantly improved germination at doses  $\geq$  350 Gy and allowed germination of larger pollen grains than those of the non-irradiated control and room humidity. Further studies are needed to confirm the efficiency of this new method *in vivo*.

The plant family Cucurbitaceae is known to exhibit a high level of endopolyploidy (Barow and Meister, 2003). Moreover, it was shown to be organ specific. Flow cytometry analysis revealed levels up to 64C in studied organs of mature styrian oil pumpkin plants. The hypocotyl, epicotyl, and cotyledon were identified as the most endoreduplicated organs, while the youngest leaf showed the lowest endopolyploidy extent. It was previously shown in cucumber that endopolyploidy can affect adventitious regeneration from cotyledonary explants (Colijn-Hooymans *et al.*, 1994). Due to the presence of starch and oil in partially etiolated pumpkin cotyledons which probably interfered with 4',6'-diamidino-2-phenylindole (DAPI) staining, we failed to analyze cotyledonary samples by flow cytometry. A suitable alternative, image cytometry, gave sufficient resolution and was therefore used for analysis. We found that the least endoreduplicated part (basal third) correlates with the highest regeneration reported in published studies (Ananthkrishnan *et al.*, 2003; Lee *et al.*, 2003; Kathiravan *et al.*, 2006; Zhang *et al.*, 2008; Kim *et al.*, 2010). Basal cotyledonary explants were then subjected to various media combinations. Among tested media supplements N<sup>6</sup>-(2-isopentenyl)-adenine (2iP), p-aminobenzoic acid (PABA) and fusaric acid (FA) were found most effective when added to N<sup>6</sup>-benzylaminopurine (BA)-based media, while Meta-topolin was the least effective. All regenerants from cytokinin-based media were diploid. Surprisingly, FA, added to media as a possible selective agent inducing increased tolerance to *Fusarium*, not only promoted regeneration at low concentrations but also induced genome doubling when medium concentrations were used. As no mixoploid regenerants were detected in our study, we believe that FA can serve as an alternative to traditional antimetabolic treatments for genome doubling. However, further studies are needed to clarify the involvement of FA in genome doubling and its efficiency across varieties and species.

The tested methods present the first step towards efficient protocols and accelerated breeding. Nonetheless, further developments are needed to ensure reliable techniques which can be applied in large scale.

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# THE EVALUATION OF PROLINE, PLANT REGENERATION AND VIABILITY AFTER *IN VITRO* STRESS SELECTION SUSTAINS DROUGHT RESISTANCE IN POTATO MARKER-FREE TRANSGENIC LINES CARRYING PVYCP INTRON CONTAINING HAIRPIN CONSTRUCT

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Potato (*Solanum tuberosum* L.) is one of the most important sources of food, being the third cultivated agricultural area in the world. It spread to all continents and is cultivated in more than 150 countries, with varied uses in food, as animal feed and also in various industries, but is very sensitive to diseases, pests and drought. From this reason, potato is one of the main targets for genetic improvement by combinatorial biotechnology.

In this case, we try to combine gene transfer with *in vitro* stress selection to obtain plants resistant to both PVY and drought stress. For this study we were considered the hypothesis that one resistance to one type of stress, can determine a state of better response to a second stress factor.

The amino acid proline is an organic osmolyte that accumulate in a variety of plant species in response to abiotic stresses such as drought. For this reason it was used to appreciate the effects of *in vitro* induced drought stress in transgenic and non-transgenic potato lines.

The callus cultures have been obtained from internodes of cvs. Baltica (control and putative transgenic lines: 1B4, 3B5, 4B9, 5B2, 5B8, 6B4, 6B8, 7B3) and Desiree (control and: 1D6, 1D9, 4D1, 6D1, 6D4, 6D6). These lines have been transformed with *Agrobacterium tumefaciens* carrying the construct PVYCP-I-PVYCP, but some did and others did not integrate the transgene in the genome. The internodes were inoculated in Petri dishes on MS-T media for three weeks. The drought stress was simulated *in vitro* on callus cultured three weeks on MS-T media with 5% PEG 6000. Then the callus with first regenerated shoots was transferred on MS-T media, without PEG. The regenerated plants after stress were evaluated for viability and proline biosynthesis, as a marker of resistance to abiotic stress.

*Proline determination.* Approximately 100 mg of *in vitro* leaf material was homogenized with 0.3 ml of 3% aqueous sulfosalicylic acid and quartz sand. The homogenate was centrifugated for 5 min to rpm max, and then 0.2 ml of supernatant was added in new tubes with 0.2 ml acid ninhydrin and 0.2 ml glacial

acetic acid and mixed, then, it was incubated for 60 min at 100°C. The reaction was stopped on ice bath, and the reaction mixture was extracted with 1ml toluene. The toluene phase was aspirated from the aqueous phase, and the absorbance at 520 nm using toluene for blank was read. The proline concentration was calculated as:  $[(\mu\text{g proline/ml} \times \text{ml toluene}) / 115.5 \mu\text{g} / \mu\text{mole}] / [(\text{g sample}) / 5] = \mu\text{mole proline} / \text{g FW}$ .

The results showed that after PEG induced stress, some regenerated shoots suffered necrosis (6D1), formed kind of runners (1D6) or even vitrified (7B3). But some of the plants with integrated PVY resistance gene showed also resistance to draught stress (4D1, 6D4, 6D6, 5B2). The line with many regenerated plants (130 plants) was 5B2.

Regenerated plants under drought stress from callus of the lines which showed a resistant character, were vigorous and showed no specific physical symptoms of water stress conditions, which demonstrates that these plants have the ability to overcome stress caused by drought conditions through its own defense mechanisms.

Analysis of proline content in plants regenerated after PEG stress showed that some of the plants with integrated PVY resistance gene showed also resistance to draught stress (4D1, 6D4, 6D6, 5B2) and had a higher concentration of proline compared with the control. The line 5B2 had the highest concentration of proline (899.34  $\mu\text{Moles} / \text{g FW}$ ) accumulated in leaf tissue, compared both with other lines and control.

Between the lines which have been transformed with *Agrobacterium tumefaciens* carrying the construct PVYCP-I-PVYCP and did not integrate the transgene in the genome but they still showed resistance to stress, just only 6B4 had a higher proline concentration compared to the control. The other lines (1D6, 1D9, 6D1, 4B9, 6B8, 7B3) had a lower concentration than the control.

It was observed a positive correlation between plant regeneration in the presence of PEG and proline biosynthesis, which revealed two groups of plants: sensitive and resistant to drought.

The results obtained reveal that it is possible that one stress factor induces general response mechanisms to an additional stress factor.

Under draught stress induced by PEG the number of viable plants per explant but also the concentration of proline accumulated in leaf tissue show that the most resistant lines were 4D1, 6D4, 6D6 and 5B2.

These results have to be further investigated in the field in order to prove the combined resistance to both PVY and draught stress of the lines selected for the cultivars Desiree and Baltica.

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# TRANSGENIC PLANTS BENEFIT FROM REACTIVE ALDEHYDE DETOXIFYING AND SUGAR ALCOHOL PRODUCING FUNCTION OF THE SAME ALDO-KETO REDUCTASE ENZYME DURING HEAVY METAL, FREEZING AND SALT STRESS

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Plants are exposed to oxidative damage under various stress conditions. A major portion of oxidative stress originates from the photosynthetic system. For instance, dark reactions of the photosynthesis can be suppressed, either as a consequence of low stomatal conductance (caused by water deficit or Cadmium treatment) or of low temperature. In these cases, light could become excess and reactive oxygen species (ROS) are formed during light reactions. These compounds not only contribute to injury of different parts of the cell *per se*, but cause indirect injuries via products of lipid peroxidation. Lipid peroxidation refers to the reaction of ROS with membrane-bound polyunsaturated fatty acids. Certain lipid peroxide breakdown products, the reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) contain two double bonds and so are able to cross-link proteins.

These compounds are effectively detoxified in many plants including e.g. alfalfa, rice and corn by stress-inducible aldo-keto reductase enzymes (AKRs). Osmotic stress also presents a common threat during many types of environmental stresses, including at least drought, freezing, and salt stress. During these conditions, plant cells may be exposed to dehydration and protein denaturation. The production of osmoprotectants e.g. sugar alcohols may improve the osmotic stress tolerance by providing osmoprotection and osmotic adjustment. On one hand, many AKRs are known to be capable of sugar alcohol production, including e.g. the celery mannose-6-phosphate reductase. On the other hand, less evidence exists for that some AKRs can both detoxify reactive aldehydes and synthesise sugar alcohols. We had already established transgenic (tr.) plant lines constitutively expressing the AKR4C9 enzyme of *Arabidopsis thaliana* origin. The AKR4C9 had been shown to be active both on reactive aldehyde and carbohydrate type substrates, glutaraldehyde and fructose, *in vitro*. Reactive aldehyde detoxifying function of AKR4C9 had also been verified *in vivo*, by glutaraldehyde treatment of intact plants. During present works, sorbitol production of the tr. plants had been studied (it was assumed from fructose reduction). Besides this, viability of tr. and control plants were studied under

stress conditions generating strong oxidative (Cadmium stress) and/or osmotic stress (salt stress, freezing stress).

The tr. barley (*Hordeum vulgare* L. cv. 'Golden Promise') plants (T3 progeny generation) carried the rice actin promoter:: *Arabidopsis thaliana* At2g37770.2 gene:: *Agrobacterium tumefaciens* nos terminator construct. Stress physiology experiments were carried out on plants grown in hydroponics. Cd tolerance was studied by applying 5 days of 10  $\mu$ M Cd(NO<sub>3</sub>)<sub>2</sub> treatment, while salt tolerance was studied by applying 6 days of 175 mM NaCl treatment. Freezing tolerance was assessed after cold acclimation and triple freezing-thawing at -20°C/+4°C.

As for Cd treatments, the high AKR4C9-expressing line, C1 was shown to have less MDA-content, than either the control or lowAKR4C9 expressing plants. Furthermore, total chlorophyll content and PSII maximal quantum efficiency ( $F_v/F_m$ ) of this line was higher than that of the controls. Cadmium tolerance of the low AKR4C9 expressing line, C2 was comparable with that of the control. Altogether, AKR4C9 was found to play a role in Cd tolerance. However its protective effect is limited, since this enzyme does not protect the plants from all aspects aspects of Cd stress (e.g. binding to sulfhydryl groups of enzymes, inhibition of iron uptake).

The sugar alcohol, sorbitol was detected both in control and in tr. plants, but its concentration in the tr. plants reached 2-4 fold of that of the control. Sorbitol production correlated well with AKR4C9 production. In the next set of experiments, control and tr. plants were exposed to stress conditions where sorbitol production might be of adaptive value. In the case of salt stress, considerable salt stress tolerance of the tr. plants has been suggested by total chlorophyll content, chlorophyll a/b ratio,  $F_v/F_m$  parameter and chlorophyll/carotenoid ratio measurements. It should be noted, that there was no increase in MDA-content of any plants. It indicates that the enhanced salt tolerance was solely the result of sorbitol production. As for freezing stress treatments, the high AKR4C9-expressing line C1 has proved to be highly frost-tolerant, based on growth parameters, electrolyte leakage and post-frost regenerative capacity. As result of frost treatments, a slight increase in MDA-content was also detected in all lines. Post-frost regeneration was therefore likely facilitated by AKR4C9, removing reactive aldehydes. Altogether, both functions of AKR4C9 might have contributed to freezing stress tolerance.

The coding gene of AKR4C9, At2g37770.2 has proved to be a valuable transgene in terms of both sugar alcohol production and stress tolerance. In explanation, many transgenes (e.g. *mtld* from bacteria) applied for sugar alcohol production, do not have, any other function. Meanwhile, expression of At2g37770.2 even enhances reactive aldehyde tolerance of the plant, and therefore transgenic plants can be protected against many aspects of oxidative and osmotic stress.

# EFFECT OF THE HARDENING UNDER DIFFERENT LIGHT CONDITIONS ON MAIZE CHILLING TOLERANCE

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Plants are exposed to many environmental stresses, which are further aggravated by the effects of global climate change. Abiotic stresses such as cold, salinity, heat, drought and UV-B radiation represent serious problems to agriculture. In response to abiotic stresses various biochemical and physiological changes are induced in plants leading to the ability of plants to cope with stress. Maize is an important crop plant which is normally grown under sub-tropical conditions. The chilling tolerance is very important, because during the early developmental stage of maize chilling can be occurred in Hungary. The aim of the present work was to investigate how the different light conditions during hardening influence the chilling tolerance of maize plants.

Norma hybrid was used for the experiments. Plants were grown in hydroponic solution at 22 °C/20 °C using 16/8 h light/dark periodicity for 10 days under 180  $\mu\text{molm}^{-2}\text{s}$  light condition. After it part of the plants were transferred to 15/13 °C for three days under different light conditions: full light (FL) was 180  $\mu\text{molm}^{-2}\text{s}$  and two lower light intensity, S1: 14  $\mu\text{molm}^{-2}\text{s}$  and S2: 46  $\mu\text{molm}^{-2}\text{s}$ . Chilling stress was carried out at 5° for 4 days at continuous full light then plants were transferred back to growing temperature for recovery for two days. Leaf and root samples were collected before hardening and chilling and after chilling and recovery. Leaf viability was measured using conductivity measurement method. For detection of the oxidative stress lipid peroxidation was detected *via* malondialdehyde (MDA) content spectrophotometrically. Leaf and root samples were used for determination of soluble sugars, namely glucose, fructose, maltose and sucrose. The analysis was carried out using an HPLC equipped with a differential refractometer.

It can be seen from the results that the leaf membrane permeability increased at FL during the hardening but the S1 and S2 was not changed. After the chilling the electrolyte leakage of FL plants did not change but the ion permeability of plants hardened at S1 and S2 increased highly. The MDA content in leaf of the FL plants increased, but the S1 and S2 decreased during the hardening. During chilling the FL increased but not as much as the S1 and S2. The content of glucose, fructose and maltose also changed in the leaves. Their content increased in the case of FL plants but decreased at lower lights. The chilling increased the sugar contents of S1 and S2 plants but not as much as the plants hardened at FL. Only the changes detected in the sucrose content was different in the leaves, because there was no change at FL but decreased at S1 and S2. Chilling increased the sucrose content in FL plants but not as much as in S1 and S2 ones.

The MDA content in root of the FL plants increased but it decreased in the S1 and S2 ones during the hardening. In contrast to it, during the chilling the MDA content of S1 and S2 plants increased but not as much as the FL ones. The content of glucose and fructose increased in the roots of FL plants but decreased in the roots of the plants hardened under low light conditions. The chilling increased the sugar content of S1 and S2 plants but not as much as the FL ones. The maltose and sucrose content did not change during the hardening and increased only in the root of the FL plant.

It can be concluded from these results that hardening can increase the chilling tolerance of maize plants and light has an important role during this process.

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# THE EFFECTS OF DROUGHT ON PLANT DEFENCE SYSTEM IN WHEAT GENOTYPES WITH DIFFERENT SALICYLIC ACID CONTENT

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Because of the continental climate aridity is a well-known and common phenomenon in Hungary. Nowadays it may occur in later spring and summer, earlier and earlier every year, when high temperature associates with low rainfall and high UV-B radiation too. This strong drought affects several physiological processes at various levels such as growth, development and limits the productivity of crops. The deterioration in quality and quantity of cereal yield leads to serious economic damages. To avoid this we focus on research aimed at crops with tolerance against different environmental stresses, including for example water deficit, UV-B radiation and other abiotic stresses.

In this study we investigated how drought stress influences the endogenous salicylic acid (SA) content, with particular emphasis on the connection between SA content and drought tolerance and even between SA and certain protective compounds during drought stress in wheat plants with different levels of endogenous SA content. SA is a well-known signal molecule which may provide protection under biotic and abiotic stress conditions. However, the exact mechanism of the mode of action of SA is still poorly understood, especially because it may differ in the different plant species, and may also depend on the environmental stress factors. The protective effect of exogenously applied SA against drought stress induced injury has been demonstrated in several plant species, and this enhanced tolerance is mainly accompanied by the increased activity of certain antioxidant enzymes. Besides reports on the protective effect of exogenous SA application, endogenous SA accumulation during drought stress has also been reported on several occasions.

Based on our preliminary field results, two near-isogenic Thatcher-based wheat lines (TC19 and TC33), with high initial endogenous SA contents, and two genotypes from Martonvásár (Mv Hombár and Mv8), with low initial endogenous SA contents, were selected. Seedlings were grown under controlled environmental conditions in growth chamber and two weeks later half of the plants were treated with 15% PEG, added into the modified Hoagland solutions for 5 days, which induced drought stress, the other half of the seedlings was the control. The first, most sensitive sign of water deficiency is a reduction in turgor, leading to the retardation of growth processes. Visual analysis of plants showed that control plants of Mv wheat

varieties had lower phenotype with narrow leaf surface, but with higher chlorophyll content compared to the Thatcher genotypes. Drought stress caused changes in the dry weight/fresh weight ratio both of leaves and roots of the investigated genotypes. Leaf rolling as a strong manifestation of leaf response to water deficit was observed in all the genotypes after 5 days of drought treatment, with the highest manifestation in TC33 and the lowest in Mv Hombár.

PEG affected plants at the physiological level and at the biochemical and molecular level also such as formation of radical scavenging compounds, accumulation of compatible organic solutes and changes in endogenous phytohormone contents. Although  $\Delta F/F_m'$  chlorophyll-a fluorescence induction parameter did not show any differences between the genotypes or the treatments, the proline content increased both in the leaves and roots after PEG treatment, with the highest accumulation in TC33. Drought stress induced changes in the antioxidant system namely, in the activity of catalase, ascorbate peroxidase, mono-dehydro-ascorbate reductase, glutathione reductase, guaiacol peroxidase (GPOD) and glutathione-S-transferase (GST), especially in the later two enzymes, and also in the level of thiol compounds (cysteine,  $\gamma$ -glutamyl-cysteine and glutathione) and polyamines indicating the induced oxidative stress. A one week recovery phase after, when treated wheats were grown in clear Hoagland solution without PEG, most of the antioxidant enzymes lost their induced activity, but not GPOD. It means enzymatic defence system induced under the stress, but when the stressor disappeared the main part of this system returned to the basal level, except GPOD, which may play role in hardening against to drought. The SA content did not change either in free or bound forms in the leaves of near-isogenic Thatcher lines, while in the wheat varieties from Martonvásár slight increases were measured after PEG treatment. Additionally, in the roots the bound SA content increased in all the investigated genotypes. Despite pronounced initial variation in the SA level were detected in the four genotypes this could not explain the observed differences in the level of tolerance to drought stress.

Based on these results it was concluded that although drought stress induced the antioxidant defense system and increased SA levels, there was no correlation between the initial levels or stress-induced changes of SA and the stress resistance levels of the plants. But there was positive correlation between certain SA-related compounds and protective compounds. It suggests that SA-related signalling may also play a role in the acclimation to drought stress; however, the direct connection is still unclear and needs further research.

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# SOLID-STATE FERMENTATION – AN ALTERNATIVE METHOD FOR ENRICHMENT OF AGRO- BASED MATERIALS WITH BIOACTIVE COMPOUNDS

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Plants and crops play significant role in human life. Of them, cereals and legumes are irreplaceable components in human diet and feed industry all over the world. The most consumed cereals comprise of wheat, barley, oats, rice, sorghum and maize. On the other hand, the main cultivated legumes are soybean, bean, lentil and pea. However, although cereals and legumes are important sources of several macronutrients (proteins, carbohydrates) and micronutrients (minerals, vitamins), they are quite low in fats lacking some essential polyunsaturated fatty acids (PUFAs) and other lipophilic compounds (e.g. pigments). Therefore, a diet based primarily on cereal grains and legumes might not only encourage an improper dietary balance of these metabolites but also it may finally lead to an increased incidence of various diseases resulting from an insufficient intake of PUFA and carotenoid pigments.

Nevertheless, cereals and legumes could be considered as challenging sources of these compounds, providing that they are naturally modified with the aim to enrich them in PUFAs and pigments. The intensive research carried out over the past several years has been aimed at agro-biotechnological techniques, such as breeding and genetic engineering. Plant breeding is the most common method for changing the traits of plants in order to produce desired characteristics. However, classical natural breeding is not useful to precisely modify metabolic pathways with the aim to synthesize new metabolites. The modern breeding employing genetic modification can result in the improvement of preferred properties of conventional cereals or legumes if their genome is appropriately altered by introduction of novel genes. Even though there are difficulties in the effective transformation of genes to cereals, the introduction of the fungal  $\Delta 6$ -desaturase gene into cereals has only recently been reported where barley and wheat containing PUFAs have been prepared<sup>[1, 2]</sup>.

Another useful and effective biotechnological process how to enrich cereals and legumes with PUFAs/pigments is based on solid state fermentations (SSF) where suitable microorganisms utilize cereals and convert them to new cereal-derived bioproducts with a high content of valuable metabolites<sup>[1]</sup>. Processes based on SSF have been well known since ancient times, especially in Asian countries where they have been mainly used for food production (koji, soybean sauce, sake,

etc.). SSF is a process in which microorganisms grow on a moist solid substrate in the absence of free water. These techniques also offer couple of advantages like utilization of low-cost agro-industrial residues as substrates, reduced costs for downstream processing, reduced production of waste water, lower energy costs, etc. Lower filamentous fungi, such as *Thamnidium* sp., *Cunninghamella* sp., *Mucor* sp. and *Mortierella* sp. are considered as the most suitable organisms for utilization of cereals and legumes due to their appropriate enzymatic equipment which allows them to penetrate inside the substrates, efficiently consume all nutrients and produce demanded metabolites. These microorganisms with an active lipid metabolism efficiently produce various PUFAs, such as  $\gamma$ -linolenic acid (GLA), dihomo- $\gamma$ -linolenic acid (DGLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA).

The first approach to enrich cereals and legumes with PUFA was focused on GLA<sup>[3]</sup>. GLA has a number of possible therapeutic applications and it has been found to be very important compound in the medical, pharmaceutical and food fields. GLA is available in small commercial quantities from the certain plant seeds (Evening primrose, blackcurrant, borage). Recently, dual production of GLA and  $\beta$ -carotene was described by various *Mucor* strains<sup>[4, 5]</sup>. After the basic strain screening performed on wheat bran and corn groats, *Mucor wosnessenskii* CCF-2606 was considered as the best strain for the simultaneous production. Cultivation with the strain was carried out on seven cereal and seven legume substrates. It was found that prefermentation of the mixture oat flakes/spent malt grains (3:1) resulted in 3.6 mg GLA and 140  $\mu$ g  $\beta$ -carotene/g bioproduct, while utilization of chickpeas yielded to 4.4 mg GLA and 85  $\mu$ g  $\beta$ -carotene/g bioproduct. In order to reach maximal productivities of the two metabolites, SSF was further optimized by addition of various concentrations of glucose, starch, glycerol and sunflower oil. GLA production increased by 1.4 times when 10% glycerol was added to cereal substrate and 3 times when oat flakes/spent malt grains (3:1) was supplemented with 20% sunflower oil (10.7 mg GLA/g bioproduct). On the other hand, the fungus effectively converted both oat flakes/spent malt grains (3:1) and chickpeas supplemented with 10% and 5% sunflower oil and the final levels of  $\beta$ -carotene in prefermented cereal and legume was increased up to 262  $\mu$ g  $\beta$ -carotene and 120  $\mu$ g  $\beta$ -carotene/g bioproduct, respectively. So, this natural biotechnological technique is promising for the application of prefermented cereal and legume bioproducts enriched with PUFAs and carotenoids might be considered as new types of inexpensive functional cereal-based food and feed supplements.

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# COLD RESPONSE AND GENE EXPRESSION PATTERN CHANGES IN TWO CHROMOSOME SUBSTITUTION LINES OF WHEAT (*TRITICUM AESTIVUM* L.)

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Low temperature is one of the most critical environmental factor during plant development, and the plant hormones, also known as phytohormones, are playing an important role during the biotic and abiotic stress response as key regulators. The aim of our research work is to elucidate the interaction of different phytohormones during the cold acclimation process and to reveal how they affect the ability of wheat cultivars to survive the inordinate climatic changes. Many gibberellin (GA) biosynthesis associated genes already identified and classified in Arabidopsis model organism and cereal mutants. GAs are a members of diterpenoid compounds of the plant metabolome. Some of them bioactive growth regulators, which are essential during control of seed germination, stem elongation and leaf expansion. The environmental factors, such as light intensity and temperature have an effect on GAs concentration which able to modify the plant development. Abscisic acid (ABA) dependent signaling pathway is one of the most important regulator system affecting the expression of many stress related genes. These ABA regulated genes are the key elements of the frost damage avoidance during cold hardening. Cold stress is able to increase the ABA level in many plant organs, but the exact role of this phytohormone in cold response is still not highly understood. Cytokinins are playing a central role in many physiological processes in plants, including development and growth regulation. It is still not well established what are their role in cold acclimation process.

Our previously constructed microarray chip contains about sixteen thousands of gene probe sets, and RNA samples of four different wheat genotypes as plant materials were used for hybridization. The plants were raised up at normal growth conditions, than they were treated with 4 °C for a day. The selection of plant genotypes were based on their growth habit and their tolerance against frost. Accordingly: one winter habit cultivar with high frost tolerance (Cheyenne), and one spring habit cultivar with medium frost sensitivity (Chinese Spring) were studied. More over two chromosome substitution lines with spring habit were involved. From these lines CS/Ch5A was frost tolerant while, CS/Tsp5A was frost susceptible.

The custom microarray was already contained an annotations about a gene functions, but it was strongly imperfect. At the meantime many new information

have been published about a wheat genome, and based on this new findings we re-annotated the microarray datasets. The reconstructed database is now containing several up to date information, such as EST sequences, SwissProt and KEGG annotations, including metabolic pathways.

For the microarray validation ten different genes from the datasets were chosen, which featured expression response to cold treatment in all genotypes compared to the non-treated samples. The oligonucleotide primer sets were designed for EST (Expressed Sequence Tags) sequences, and the proposed amplicon length and qPCR conditions were adjusted to regular settings. The gene expression changes were evaluated by the  $\Delta\Delta\text{Ct}$  method. The studied ten genes showed a very similar treatment dependent expression changes like in the previously constructed DNA chip.

The phytohormone metabolites were extracted from cold treated and control leaves and crowns, then their contents were measured by HPLC. Two of the cytokinin compounds were differently accumulated in the chromosome substitution lines, compared to wild type genotypes. The results suggest that the trans-zeatin level were higher after 1 day 4 °C cold treatment both in leaves and crowns, while the dihydrozeatin increased significantly only in the CS/Ch5A line. ABA and two ABA-related intermedier compounds, like dihydrophaseic acid and neophaseic acid were less accumulated in the CS/Ch5A and CS/Tsp5A lines independently of plant organs.

The studied genes were chosen from the microarray, including zeatin and ABA biosynthesis pathways which were also containing the previously measured hormone metabolites. We measured the expression of *isopentenyltransferase 8* gene (*TaIPT8*) from the zeatin biosynthesis pathway, but we could not found any correlation between the gene expression profile, metabolite quantity, treatment and genotypes. Furthermore the *9-cis-epoxycarotenoid dioxygenase* gene (*TaNCED3*) expression was correlated with the accumulated ABA level, both in leaves and crowns. Our advanced result are suggesting that the chromosome substitution wheat lines, especially the CS/Ch5A genotype has a defined hormone biosynthesis modification at the function of cold treatment compared to the other genotypes and the control conditions.

The microarray probe sets and EST sequences were localized in different wheat chromosomes based on the EnsemblPlants nucleotide scaffold database. However these already sequenced scaffolds still not completely assembled, we have been found putative chromosome 5A localized genes. We used MapMan analysis which have allowed to observe changes in gene expression patterns in many pathways at the function of wheat genotypes.

Our further plan is to building up the complete expression profile of the phytohormone biosynthesis related genes, based on our previous results and a newly constructed cereal protein interaction database.

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# UTILIZATION OF DIPLOID WHEATGRASSES FOR ALIEN CHROMOSOME DISCRIMINATION IN WHEAT × AGROPYRON GLAEL HYBRID PROGENIES

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Useful genetic resources can be found among uncultivated plants in the wild. *Thinopyrum* species are excellent sources of leaf rust and stem rust resistance. Since their initial hybridization with wheat about 70 years ago, a number of resistance genes against wheat diseases have been transferred from the wheatgrasses into wheat in form of wheat-*Th. ponticum* and wheat-*Th. intermedium* chromosome translocations.

*Agropyron glael* is a hybrid of *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey (JJ<sup>st</sup>St, 2n=6x=42) and *Thinopyrum ponticum* (Podp.) Z.-W. Liu & R.-C. Wang (JJJJ<sup>st</sup>J<sup>st</sup>, 2n=10x=70) produced by Tsitsin in the first half of 20th century. It contains excellent resistance against biotic and abiotic stresses.

*Triticum aestivum* (Mv9kr1) and *Agropyron glael* (2n=8x=56) hybrids were produced in Martonvásár in 2001 and were backcrossed with wheat in order to incorporate the advantageous traits of *A. glael* into cultivated wheat. The genome constitution of the original *A. glael* is not known. Multicolour GISH technique was used to detect parental genomes of the *Agropyron glael*. Two genomes are labelled during mcGISH simultaneously, the GISH parameters for *A. glael* was elaborated in the present study. The parental species of *A. glael* (*Thinopyrum ponticum*, *Thinopyrum intermedium*), *Agropyron glael*, the Mv9kr1 × *A. glael* F<sub>1</sub> hibrid, and BC<sub>1</sub> and BC<sub>2</sub> progenies were analyzed using mcGISH.

Seeds of *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey and *Thinopyrum ponticum* (Podp.) Z.-W. Liu & R.-C. Wang were germinated and mitotic metaphase chromosome spreads were prepared. *Th. intermedium* and *Th. ponticum* contains similar genomes to wild diploid wheatgrasses. Total DNA from these species (*Elytrigia elongata* (2n=2x=14, EE) *Thinopyrum bessarabicum* (2n=2x=14, JJ) and *Pseudoroegneria spicata* (2n=2x=14, StSt)) was labelled with biotin-16-dUTP or digoxigenin-11-dUTP using the random primed labelling protocol. In case of *Th. ponticum* J + St and E + St probe combinations were used. The hybridization mixture contained 60ng each of the labelled probes dissolved in a 15µl mixture of 100% formamide, 20xSSC and 10% dextran-sulphate. In order to denature the probe DNAs, the hybridization mixture was heated to 75 °C for 6 minutes and then immediately cooled on

ice. The chromosome spreads were denatured at 80°C in a high formamide concentration mixture containing 100% formamide, 20xSSC and 10% dextran-sulfate. Hybridization was performed at 42°C overnight. Streptavidin-FITC and Anti-Digoxigenin-Rhodamine dissolved in TNB were used in the detection phase. Finally, the chromosome spreads were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in an antifading solution. The slides were screened using a Zeiss Axioskop-2 fluorescence microscope equipped with filter sets appropriate for DAPI, FITC, Rhodamine, and for the simultaneous detection of FITC and Rhodamin. Images were captured with a Spot CCD camera and processed with Image Pro Plus software.

There were no differences between the hybridization pattern and intensity of J and E genomic DNA probe in *Thinopyrum ponticum*, confirming the theory that J and E genome is the same. The origin of J<sup>st</sup> genome is not clear. St genomes can be found near the centromeres, and J genomic DNA hybridized to the other part of the chromosomes but with less intensity than that of J (=E) chromosomes. Altogether 28 J<sup>st</sup> and 42 J(=E) chromosomes were detected, showing precise concordance with the JJJJ<sup>st</sup>J<sup>st</sup> genomic formule. JJ<sup>st</sup>S genomic composition was not proved in *Thinopyrum intermedium*, as 19 J, 9 J<sup>st</sup> and 14 St chromosomes were detected. 14 J, 14 J<sup>st</sup> and 14 St was the expected chromosome number. This phenomenon had also described by other cytogenetic groups.

All genome related (J, J<sup>st</sup>, St) chromosomes were detected in *Agropyron glael* and F<sub>1</sub> hybrid of Mv9kr1 (wheat) × *A. glael*. Large number of *Agropyron-wheat* and *Agropyron-Agropyron* translocations were detected in the BC<sub>1</sub> progeny generations. Alien chromosome number of these lines varied between 15-19. St chromosomes were eliminated more rapidly than others, but wheat/St genome translocations were visualized in some cases. Cytologically analyzed plants were grown up in phytotron chamber. BC<sub>1</sub> derivatives were leaf rust resistant, but the plants were more similar to the *Agropyron* parents (cca 120 cm plant height, long spike, late flowering), which is not advantageous for wheat breeding. The derivatives were grown up in the nursery every year. The resistant and susceptible lines were separated considering the spontaneous leaf rust infection data. In order to decrease the number of the alien chromosomes in the wheat background, the leaf rust resistant lines were succesfully backcrossed with wheat producing BC<sub>2</sub> generations.

The reduction of alien chromosome number (2-14) was observed in BC<sub>2</sub> progenies. Derivatives with high chromosome number were not stable. Elimination of wheat chromosomes 3D were detected in one line using fluorescence *in situ* hybridization (FISH). The presence of alien chromosome (segment) could not be detected in BC<sub>3</sub> progeny plants. Wheat/*Agropyron* translocations and introgression lines with 2-4 alien chromosomes were managed to sort out among the BC<sub>2</sub> progenies by the end of last year. Detection of new introgressions and identification of the chromosomes are in progress.

Our final aim is to select and identify leaf rust resistant lines which carry only one pair of alien chromosomes or just a segment.

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# NOVEL UTILIZATION OF WILD RELATIVES IN BREAD WHEAT PREBREEDING

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As the negative effects of the global climate change are increasing, it is vital to improve the bread wheat (*Triticum aestivum* L.) breeding lines continually, adorning them with better resistance that can cope with the main biotic and abiotic stresses. One possible way for improving the resistance and adaptability of bread wheat is the utilization of its wild relatives in prebreeding. Most studies on the utilization of the genes responsible for resistance have involved direct crosses between *T. aestivum* and its wild relatives. However, apart from direct crosses, another possible way of utilization could be the development of new synthetic amphiploid wheat lines. Present study is focusing on the simultaneous utilization of *Triticum timopheevii* Zhuk. ( $2n=4x=28$ , A<sup>1</sup>A<sup>1</sup>GG) and *Triticum monococcum* L. subsp. *monococcum* (einkorn;  $2n=2x=14$ , A<sup>m</sup>A<sup>m</sup>) through their hybrid, because both of them have outstanding resistance against the main wheat fungal diseases.

Before crossing it is necessary to choose the most suitable lines from these species, in order to gain valuable prebreeding materials. Therefore, detailed characterisation was carried out on all of the 56 *T. timopheevii* accessions of the Martonvásár Cereal Gene Bank for the main phenotypic and resistance characters. The evaluation of the assessment data revealed that members of the base species and subsp. *timopheevii* are likely to be more advantageous for wheat breeding than those of the subsp. *armeniicum* group. Although the latter have relatively early heading date, they are more susceptible to fungal and viral diseases, and have a prostrate growth form with lower yielding ability and brittle rachis, which could make their utilization in wheat breeding more difficult. After a detailed evaluation of the results, the best 11 accessions from the ssp. *timopheevii* group were selected for a crossability test with an already prebred, semi-dwarf einkorn line, '1T-1'. On the basis of the results one accession (*Triticum timopheevii* Zhuk. var. *rubiginosum*, Acc. No.: MVGB845) with the best seed set was selected for the development of the new synthetic amphiploid, *Triticum timococcum*. After crossing, the triploid genome ( $2n=3x=21$ ) of the selected hybrid seedlings ( $F_1$ ) was doubled by colchicine-treatment ( $F_1 \rightarrow C_1$ ).

Detailed phenotypic evaluation was carried out under natural and artificial growing conditions in 2012 and 2013. Based on the results, *T. timococcum* has intermediate characteristics in terms of spike (spikelet) shape and plant height, while it is similar to the female parent, *T. timopheevii* regarding pubescence. Like its parents, *T. timococcum* showed outstanding resistance to the main fungal diseases of wheat. *T. timococcum* headed later and developed longer and looser spikes, fewer

tillers and only a third as many seeds than its parents. Beside the field assessment, an additional trial on artificial leaf rust infection of seedlings has also proved the high resistance of *T. timococcum*.

Identification of the hexaploid genome of this synthetic amphiploid using fluorescent *in situ* hybridisation (FISH) with repetitive DNA probes has proved normal doubling with 42 chromosomes in most of the plants of the C<sub>2</sub> and C<sub>3</sub> generations. Detailed identification has revealed that this plant material has 14 chromosomes originated from einkorn, and 28 chromosomes originated from *T. timopheevii*. This result was also confirmed by genomic *in situ* hybridization (GISH), which helped to effectively distinguish the A and G genomes. Genomic DNA of the S genome was extracted from the diploid *Aegilops speltoides* Tausch, because this genome is similar to the G genome of *T. timopheevii*. In the case of A genome, DNA was extracted from *Triticum urartu* Thumanian ex Gandilyan, the A genome donor of bread wheat, because the A genome of *T. timopheevii* is mostly similar to that of wheat. The successful discrimination of the chromosomes was supported by the karyotypes of the parental genomes and the successful optimization of the GISH technique for the A<sup>m</sup>, A<sup>t</sup> and G chromosomes was achieved in the present study.

The simultaneous utilization of *T. timopheevii* and *T. monoccoccum* has continued with the development of hybrids between *T. timococcum* and *T. aestivum* 'Mv9kr1' (carries the recessive allele of kr1 crossability gene) followed by back-crosses with Mv9kr1 in order to get more useful materials for wheat breeders. A comparative crossing trial proved that crossing with *T. timococcum* is less effective than the direct cross between bread wheat and *T. timopheevii*. However, using *T. timococcum* could help in the introduction not only of *T. timopheevii*-derived, but also of einkorn-derived genes into bread wheat.

Progenies of the first back-cross (BC<sub>1</sub>) were also examined with FISH and GISH according to the findings of the fluorescent *in situ* hybridization of *T. timococcum*. Beside several G chromosomes, some A<sup>m</sup> chromosomes were also identified in the bread wheat genetic background of the BC<sub>1</sub> plants showing promising opportunity for the development of different addition lines and/or translocations after the further back-crosses. The second back-cross (BC<sub>2</sub>) was successfully carried out in the beginning of 2014. The progenies will be grown in climate chamber, and their genomes will be examined soon.

Study has also begun on the molecular cytogenetic identification of *T. aestivum* 'Mv9kr1' × *T. timopheevii* subsp. *armeniicum* hybrids developed earlier in Martonvásár. Three backcrosses with Mv9kr1 were carried out on this material at that time. Present study has started to analyse the genome composition of these BC<sub>3</sub> lines using improved molecular cytogenetic techniques (FISH, GISH), and some promising results have already been obtained. One of the lines was identified as a disomic addition line, which is carrying the pair of 6G chromosomes of *T. timopheevii*, and this line showed outstanding resistance against leaf rust in an artificial inoculation trial carried out on seedlings.

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# DEVELOPMENT AND IDENTIFICATION OF NEW WHEAT-BARLEY DITELOSOMIC ADDITION LINES USING FLUORESCENCE *IN SITU* HYBRIDIZATION AND MOLECULAR MARKERS

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The intergeneric hybridization of wheat and barley makes it possible to transfer agronomically useful traits (e.g. earliness, drought tolerance, salt tolerance or nutritional parameters) from barley into wheat. It is necessary to produce wheat-barley addition and introgression lines with agronomically adaptable six-rowed winter barley cultivars to utilize these traits. The wheat/barley ditelosomic addition lines are useful to determine the chromosome arm localization of genes responsible for certain agronomic traits and are good starting material for production of centric fusions. They can provide an excellent genetic material for chromosome flow-sorting, which makes possible the isolation of chromosome specific repetitive or unique DNA sequences.

A wheat × barley hybrid was produced in Martonvásár from a cross between a Japanese facultative wheat cultivar 'Asakaze komugi' (Asakaze) as female and a Ukrainian six-rowed winter barley cultivar 'Manas' as male. The BC<sub>2</sub> plants originated from 'Asakaze komugi' × 'Manas' hybrids contained different barley chromosomes. Ten ditelosomic addition lines were selected from the progenies of self fertilized BC<sub>2</sub> plants (2HS, 2HL, 3HS, 3HL, 4HS, 4HL, 6HS, 6HL, 7HS, 7HL) using genomic *in situ* hybridization (GISH). The addition lines were identified by fluorescence *in situ* hybridization (FISH) using repetitive DNA probes (HvT01, GAA, pTa71, Afa family and centromere specific AGGGAG probe). The cytogenetic identification of the ditelosomic addition lines was confirmed using barley arm specific SSR and STS markers. The used molecular markers were: HvCSLF4 (for the 2HS arm), Bmag 0125 (2HL), HvLTPPB (3HS), HvM60 (3HL), HvM40 (4HS), HvM67 (4HL), Bmac0136 (6HS), Ebmac0806 (6HL), Bmac0031 (7HS) and HvCSLF6 (7HL).

The wheat/ barley ditelosomic addition lines were developed from 860 analyzed progenies of selfed BC<sub>2</sub> plants, mainly from monosomic addition lines, between 2010-2013. The plants carrying the 3HS telocentric chromosomes were selected from a 3H disomic addition line, while the 6HS, 6HL, 7HS, 7HL lines were developed from a double monosomic plant. Most of the plants were selected from the BC<sub>2</sub>F<sub>3</sub> generation (2HS, 2HL, 3HS, 4HS, 4HL, 6HS), three lines (6HL, 7HS, 7HL) from the

BC<sub>2</sub>F<sub>2</sub> generation, while the 3HL line was selected from the BC<sub>2</sub>F<sub>4</sub> generation. Thirty percent from the analyzed plants (260) were homozygous telosomic individuals, the barley chromosomes were eliminated from 46% (395) of the plants. Among the analyzed individuals monotelosomic additions, Robertsonian translocations, mono- and disomic additions, isochromosomes were found.

Ten individuals from each ditelosomic addition line were grown in phytotron chambers in order to describe the morphological characters of the plants. The germination ability of the lines was 100%, except the 7HS line where only 77% from the seeds germinated. According to the foregoing cytological analysis the stability of the ditelosomic addition lines was higher than 60%, except the 6HS line, where only 6 plants were homozygous from 20 analyzed seeds originated from a ditelosomic individual (31%). The 3HS and 3HL ditelosomic addition lines were much more stable than the 3H disomic addition line (this disomic addition was the less stable, its stability was lower than 50%).

The ditelosomic additions, the disomic additions and the parental wheat and barley cultivars were grown in phytotron chambers (Conviron GB-48) in Martonvásár. Vernalization was carried out at 4 °C for 6 weeks. The spikes of the ditelosomic addition lines are different from the corresponding disomic additions. Five lines have spikes with awn stubs (3HS, 4HS, 4HL, 6HS, 6HL), the others have awnless spikes (2HS, 2HL, 3HL, 7HS, 7HL). The plants started flowering on the 54th day after plantage. The earliest flowering lines were the 3HS, 7HS and 7HL, the last – the 6H and 6HS. In point of flowering time the results correspond to the disomic addition lines, where the 7H addition is the earliest flowering line, while the 6H is the last.

During the multiplication of 7HL line some plants were found which had very short spikes and produced very few seeds compared to the other plants from the same line. The mitotic chromosome spreads of these plants were analyzed and we concluded that each of them contained the two barley telocentric chromosomes, but the 4B wheat chromosome was broken.

All the ditelosomic addition lines are fertile, the plants were harvested and now are under processing.

Among our long term research plans is to multiply these lines in field, to analyze the agronomic traits determined by the presence of barley telocentric chromosomes in wheat background, to cross the ditelosomic addition lines with wheat ditelosomic lines in order to obtain centric fusions which are more stable than the addition lines and can be a useful material for prebreeding.

# RESTRICTION FRAGMENT LENGTH POLYMORPHISM MAPPING OF FLAX GROWN IN RADIO-CONTAMINATED CHERNOBYL ENVIRONMENT SUGGESTS THE STABILITY OF DELTA-12 DESATURASE AND FATTY ACID DESATURASE 3C GENES

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The Chernobyl area represents an unique open field laboratory allowing to observe growth and reproduction of plants adapted to the environment contaminated with radionuclides <sup>137</sup>Cs and <sup>90</sup>Cs. In the present study, flax (*Linum usitatissimum* L., variety Kyivskyyi) was grown in two experimental fields located in radio-contaminated and non-radioactive Chernobyl area. Radio-contaminated field is located approximately 5 km from the Chernobyl Nuclear Power Plant, near the village Chistogalovka, with the soil radioactivity  $20650 \pm 1050$  Bq.kg<sup>-1</sup> of <sup>137</sup>Cs and  $5180 \pm 550$  Bq.kg<sup>-1</sup> of <sup>90</sup>Sr. Non-radioactive field is located directly in Chernobyl town and have soil radioactivity  $1414 \pm 71$  Bq.kg<sup>-1</sup> of <sup>137</sup>Cs and  $550 \pm 55$  Bq.kg<sup>-1</sup> of <sup>90</sup>Sr.

The aim of this study was to characterize fatty acid desaturase (*FAD*) genes that play an important role in fatty acids biosynthesis. The delta-12 desaturase (*FAD2*, DQ222824.1) and a first part of the novel fatty acid desaturase 3C (*FAD3C*, HM991836.1) genes were analyzed using restriction fragment length polymorphism (RFLP) technique. During fatty acids desaturation, the *FAD2* gene converts oleic acid (18:1) to the linoleic acid (18:2) by formation of a second double bound (Ohlrogge, Browse, 1995). The *FAD3C* gene is involved in the conversion process of linoleic acid to the  $\alpha$ -linolenic acid (18:3), but a major role of the novel *FAD3C* gene has not been confirmed (Banik *et al.*, 2011).

The *FAD2* (1137 bp) gene and the first part of *FAD3C* gene (1785 bp) were amplified in reactions mixture contained Thermo Scientific Dream *Taq* PCR Master Mix (2 $\times$ ); Dream *Taq* DNA polymerase is supplied in 2 $\times$  Dream *Taq* buffer; dATP, dCTP, dGTP and dTTP (0,4 mmol.dm<sup>-3</sup> each); 4 mmol. dm<sup>-3</sup> MgCl<sub>2</sub>, 400 nmol.dm<sup>-3</sup> of each primer and 50 ng of genomic DNA. Amplification was performed under following

conditions: 95 °C 5 min; 95 °C 1 min, 62 °C 1 min, 72 °C 2 min, 38 cycles; 72 °C 10 min. For restriction analyses were applied the following restriction endonucleases – *BsaI*, *Acil*, *Hpy188I* for *FAD2* gene and *MnII*, *NlaIII*, *Hpy188I*, *FatI*, *Acil*, *BamHI*, *HpaII*, *EcoRI* and *MseI* for the first part of *FAD3C* gene. Digested fragments of *FAD2* and *FAD3C* genes were electrophoretically separated in 3% agarose and in 8% polyacrylamide gels. Electrophoresis was conducted at a voltage of 60 V for 6 h/polyacrylamide gels and 60 V for 3 h/agarose gels. Electrophoreograms were processed with documentation system G:Box in GeneSnap program – product version: 7.09 (Syngene).

The RFLP method was applied as a starting point to evaluate the impact of radiation stress on *FAD2* and *FAD3C* genes in flax. For both gene sequences, no association between radio-contamination and the changes of *FAD* genes on the genomic level was detected. Preliminary results suggest the stability of *FAD* genes in flax despite the long-term effect of ionizing radiation in environment. These conclusions will provide a basis for further more comprehensive genomic analyses of flax grown in the radioactive Chernobyl area.

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# STRIGOLACTONES AND PARASITIC WEEDS

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The parasitic weeds of *Orobanche*, *Phelipanche* and *Striga* spp. are causing enormous yield losses in agriculture of many countries. *Striga hermonthica*, *S. asiatica*, *S. gesneroides* and *S. aspera* are threat for food security mainly in Africa; they negatively influence the life of millions of people. *Orobanche* and *Phelipanche* spp. are causing huge losses in production of tomato, tobacco, rapeseeds, lettuce, potato, carrot, many legumes and sunflower in southern and western part of Europe, in the Middle East and India.

These parasitic plants are hemi-, or holoparasites, and they obtain all nutrients and water from the roots of host plants. The small seeds contain nutrient reserves for a few days after germination only. Therefore they developed specific safety mechanism to ensure germination at the right time at close vicinity of the right host. The seeds of most parasitic weed species germinate after warm and moist stratification period, during which primary or secondary dormancy is released and seeds become responsive to the specific compounds exuded by the roots of host plants (Matusova *et al.* 2004). There are several classes of compounds able to induce germination of parasitic plants. The most attention is given to strigolactones (SLs), which are synthesized via carotenoid pathway (Matusova *et al.*, 2005). SLs are involved in increased branching of arbuscular mycorrhizal fungal hyphae (Akiyama *et al.* 2005) and together with other plant hormones are influencing plant root and shoot morphology (Gomez-Roldan *et al.*, 2008, Umehara *et al.*, 2008). Nowadays, more functions of SLs in plants and interactions with other organisms are discovered and are under investigation.

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# MOLECULAR DIVERSITY AND PHYLOGEOGRAPHY OF BEGOMOVIRUSES INFECTING VEGETABLE CROPS IN INDIA

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Viral diseases on crop plants are major havoc for the reduction in the yield, both qualitatively and quantitatively. Small genome size and ability to multiply within host cell have made virus particles extremely dynamic and diverse.

Geminiviridae constitute an important group of plant pathogens with genomes of ssDNA and is characterized by particle morphology of twinned incomplete icosahedra and a genome comprised of circular, single-stranded DNA. Whitefly-transmitted geminiviruses (genus Begomovirus) are credited with most complex genome organization among the Geminiviruses. Begomoviruses infect variety of plants of economic importance including tomato, chilli, legumes, okra etc. in tropical and sub-tropical region. Hence, begomoviruses are greater menace in the agriculture due to new and resistant biotype of whitefly.

We studied the phylogeography and molecular mechanism/s of begomoviral infection on French bean, tomato, and guar from India. Understanding of molecular and functional aspects of viral infection on these vegetable crops will result in formulating better resistant strategies to such infection without compromising nutritional quality or yield.

## French bean (*Phaseolus vulgaris* L.)

French bean is one of the most important vegetable crops of India which is consumed widely due its exceptional nutritional value. Our aim was to determine diversity of begomoviral genome associated with bean dwarf mosaic disease (BDMD). We have cloned and sequenced begomoviral genomic components associated with bean dwarf mosaic disease (BDMD) manifested leaf samples from Varanasi and Bangalore. Sequence analysis showed bipartite nature of begomovirus. DNA A (KC019304) and DNA B (KC019305) component of virus from Varanasi sample are of 2745 and 2669 bases respectively. DNA A (KC019306) and DNA B (KC019307) component of virus from Bangalore sample are of 2735 and 2677 bases respectively. DNA A component of virus from Varanasi sample shows maximum nucleotide identity with Mungbean yellow mosaic India virus segment DNA A (FM208836), while that from Bangalore sample shows maximum nucleotide identity with Horsegram yellow mosaic virus segment DNA A (AM932427). Comparison of DNA B segment from Varanasi sample shows maximum nucleotide identity with Mungbean yellow mosaic India virus segment DNA B (AY939925), while that from Bangalore sample shows maximum nucleotide identity

with Horsegram yellow mosaic virus segment DNA B (AM932426). Thus, two distinct virus cause disease in French bean grown in geographically separated area.

#### Tomato (*Solanum lycopersicum* L.)

Tomato is widely grown and consumed vegetable crop in India and throughout the world. Tomato leaf curl disease (ToLCD) is the major catastrophe for the production of tomato. In this study, we aimed to characterize novel begomovirus species and its cognate beta satellite that produces severe symptoms of ToLCD in tomato plants from Gujarat, India.

We cloned and sequenced viral DNA from the collected samples. Sequence analysis of DNA-A (2,753 nt) showed the highest identity (87.8%) with Tomato leaf curl Kerala virus-[India: Kerala: 2008] (EU910141). Recombination analysis showed that begomovirus DNA likely to have originated by recombination between Tomato leaf curl Kerala virus and Tomato leaf curl Karnataka virus. DNA-β satellite (1,365nt) and showed the highest identity (75.6%) with Tomato leaf curl betasatellite [India: Ludhiana: 2004] (ToLCB-[IN: Lud: 04]) (AY765255). As per ICTV species demarcation criteria, it was considered as a new begomovirus species [Tomato leaf curl Gandhinagar virus (ToLCGNV) (KC952005)] and new betasatellite [Tomato leaf curl Gandhinagar betasatellite (ToLCGNB) (KC952006)]. Agro-inoculation studies in *N. benthamiana* and tomato plants confirmed that betasatellite is not only essential for symptom expression but also equally essential for accumulation of helper viral DNA in a host plant.

#### Guar (*Cyamopsis tetragonoloba* L. Taub.)

Guar, also known as cluster bean, is an annual crop belongs to *Fabaceae* (Leguminosae) family. In the monsoon 2011 and 2012, farmers' fields were surveyed to access molecular diversity of begomoviral infection on Guar plant. We were able to clone and sequence the genomic components of viral DNA. Genome organization of all isolates is as of typical monopartite begomoviruses associated with cognate beta satellite. This study shows clear picture of virus transmission from the natural host (*viz.* tomato, croton, cotton or papaya) to unrelated host (Guar). This is the first organized attempt to access molecular diversity of begomoviruses infecting Guar.

Altogether, the study reveals following information for begomovirus infection. BDMD disease in French bean is caused by bipartite begomovirus, where two different viruses infect to French beans in different geographical location. A new, recombinant and previously uncharacterized strain of ToLCV and its associated betasatellite is responsible for severe symptoms associated with ToLCD. In case of Guar, our studies confirm that, weeds are reservoir of viruses and they transmit the healthy virus and hence disease in the next cropping cycle. Different helper viral components with satellite molecules form complete disease complex, and are ultimately responsible for diverse viral symptoms in the field.

# ANALYSIS OF NSS PROTEIN OF HUNGARIAN RESISTANCE-BREAKING TOMATO SPOTTED WILT VIRUS (TSWV) ISOLATES FROM PEPPER

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*Tospoviruses*, the only plant-infecting member of the *Bunyaviridae* family, are able to infect several plant species, including vegetable, fruit and ornamental plants, causing serious damage and represent one of the most economically significant plant pathogen group worldwide. *Tomato spotted wilt virus*, the type member of the genus is one of the most important plant pathogen. TSWV is transmitted by insects belonging to the order *Thysanoptera* and the most effective vector of TSWV is the western flower thrips (*Frankliniella occidentalis*).

The spherical virus particles are 80 to 120 nm in diameter, and are surrounded by an envelope consists of host-derived lipids with two viral glycoproteins, Gn and Gc. The RNA genome composed of three single-stranded RNA molecules, which are negative or ambisense, designated long (L), medium (M) and small (S). The L-RNA encodes the RNA-dependent RNA polymerase (RdRp) that is associated with virus replication. NSm and NSS are non-structural proteins encoded by the viral M and S RNA respectively in sense orientation. NSm is the movement protein and NSS was shown to suppress gene silencing in plant host (Takeda *et al.*, 2002, Bucher *et al.*, 2003).

Management of TSWV control was first directed against the thrips using different insecticides and against weeds as host plants of the virus as well. Because of the large importance of the virus, the search for the natural resistance genes in breeding programmes has received increasing importance. Two single dominant resistance genes available for commercial resistance breeding have been described. Sw5 was identified in *Solanum peruvianum* and the Tsw gene (Jahn *et al.*, 2000) was described in *Capsicum chinense* and has been introgressed into *Capsicum annum* cultivars. Tsw resistance gene (Black *et al.*, 1996) was applied in different types of pepper (conical white, long pale green hot and sweet, tomato shape, spice pepper and blocky types) (Csilléry unpublished). Pepper cultivars carrying Tsw resistance gene upon TSWV inoculation elicit necrotic local lesions on the inoculated leaves but systemic virus infection never occurs.

In 2002, Roggero *et al.* (2002) observed systemic virus symptoms on resistant pepper cultivars in Italy. Margaria *et al.* (2004) described the same symptoms in Spain on pepper carrying the Tsw gene. The first detection

of resistance breaking strain in Hungary was only in 2010 (Salamon *et al.*, 2010), but the accurate molecular characterisation of this strain did not occur. In recent years, the resistance breaking TSWV strain caused heavy infection in the Hungarian pepper growing areas. Since the RB strains appeared previously in other countries the question was whether the different resistance breaking strains evolved independently or spread for example by propagation material.

Despite of the importance of the pathogen, Hungarian TSWV isolates have not been characterized and there was no information about the main component of TSWV epidemics. Previous works predicted the *Tsw* resistance breakdown determinant for the NSs protein of S-RNA (Jahn *et al.*, 2000, Margaria *et al.*, 2007). Therefore, the aim of our study was to characterize the NSs region of Hungarian resistance breaking TSWV isolates and to compare them to the NSs protein of other TSWV isolates.

Five TSWV isolates were included in this study, two resistance breaking isolates were collected from pepper in Szegvár in 2012 and three normal Hungarian TSWV isolates were collected from pepper in Szentes in 2005, 2009 and 2012 and were propagated on *N. tabacum* cv. Xanthi-nc. Total RNA was extracted from leaves of *N. tabacum* cv. Xanthi-nc plants systemically infected with TSWV. Total RNA was used for first-strand cDNA synthesis and subsequent RT-PCR amplification of NSs gene. The amplified fragment was cloned into pGEM T-easy vector and the positive clones were sequenced. The amplified NSs fragment was 1404 bp long. The Hungarian isolates were compared to other isolates from the GenBank on the basis of nucleotide sequence of the NSs region and a phylogenetic tree was prepared. Analysis of the NSs region revealed that the European isolates could be divided further groups based on the geographic location. The Spanish, Italian and American isolates from NCBI formed well separated subgroups and the Hungarian samples as well. Hungarian TSWV isolates collected in the year of 2009 and 2012 were in a same branch with Bulgarian isolates, while one of the Hungarian isolates from 2005 belonged to the French subgroup.

In the analysed region there was no significant difference between the resistance breaking and non-breaking TSWV strains on the basis of the nucleotide sequences (only 1-2%). In the amino acid (aa) comparison two aa variation was identified characteristic for the resistance breaking isolates, at the position of 104 and 462 the threonine was changed to alanine. Previous studies described the same mutation of the 462 position (Tentchev *et al.* 2011). De Ronde *et al.* (2014) proved that the NSs region between 370 to 467 position is responsible for RNA silencing suppressor (RSS) activity but not for resistance breaking in the case of the Brazil strain. These results suggest that the TSWV resistance breaking strains have been emerged independently and the molecular basis of the resistance breaking can be different. Further studies are required for the analysis of the detailed mechanism of resistance breaking.

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# EFFECT OF MICROALGAE LEAF TREATMENTS ON SUNFLOWER GROWTH AND PRODUCTION

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Sustainable development and environmental management play a significant role in conventional agricultural production. Global warming and extreme climatic conditions such as dryness, higher average temperatures result in an increasing challenge for plant growers. It has been established that the effects of environmental stress can be decreased in plants treated with microalgae. The production of oil plants has increased by 6-8% in recent years with sunflowers exceeding 26 million ha (Faostat, 2013). Nowadays, sunflower has become one of the most widely planted crops in Hungary with 598000 ha planted in 2013. The aim of the present research was to determine the optimum spraying time and microalgae concentration for sunflowers.

Algae cells produce and secrete intra- and extracellular compounds to the environment during their life cycle. Primary metabolites are essential for algal growth and reproduction. Secondary metabolites are necessary for maintaining the relationship between algae cells and the environment. Some secondary metabolites have allelopathic effects (Boussiba, 1988). Other metabolites are plant growth regulators (PGRs) (Erdei, 2008). Jacobs *et al.* (1985) proved the IAA (indol-3-acetic acid) content of green algae with analytical methods for the first time. Sergeeva *et al.* (2002) confirmed that naturally occurring symbiont cyanobacteria are also IAA producing organisms. IAA can be considered as the most physiological active form of auxin (Erdei, 2008). Its principal effect is the stimulation of cell elongation. Ördög *et al.* (2004) detected cytokinins in microalgae. Cytokinins regulate plant cell division and stimulate cell elongation in conjunction with auxins. Nowadays there is no doubt that microalgae produce similar plant hormones which act in the higher plants.

The yield surplus originated from the treatment of microalgae biomass is hindered by abiotic environmental factors and applied agricultural techniques (e.g. usage of herbicides and pesticides). Reliable positive effect can only be anticipated from plant treatments under well elaborated plant production technology (Singh *et al.*, 1988). Microalgae with plant protecting and growth regulating effects can be successfully used in plant treatments (Ördög and Pulz, 1995).

Trials were set up in 2013 in Mosonmagyaróvár. The soil type of the experimental area is multi-layered humic, river-terrace soils. The upper 30 cm layer of this

soil type has the following characteristics: humus content=3,09%, a  $K_a=45$ ,  $pH_{H_2O}=8.09\%$ ,  $pH_{KCl}=7,40$ .

The sowing date was on 10<sup>th</sup> April 2013. The plot size was 27 m<sup>2</sup> (4,5×6m) with 6 rows of plants. The net plot size included the middle 4 plant rows which were used for harvesting samples. The row distance was 75 cm, the stock distance 24 cm, and the number of stocks were 55.000 achene/ha. Weed control was performed mechanically during the growing season. There were 7 treatments (including control) in 4 replications and arranged in a randomized block design. There experiment included 28 plots.

Sunflower (*Helianthus annuus* L.) cv. "Nk Neoma" was treated with *Nostoc entophyllum* (MACC-612) and *Tetracystis* sp. (MACC-430), which originated from the Mosonmagyaróvár Algal Culture Collection (MACC). The first treatment was applied at the 4-6 leaf stage and the second treatment at the rosette growth stage of sunflower growth. Plots were treated with biomass of the MACC-612 and MACC-430 in a dosage of 400 and 700 g/ha (in a concentration of 0.1%). Spray amounts were 400 and 700 l/ha in the first and second treatment, respectively.

Plant height was measured just before the first and second treatment and after treatments every 10 days. Measurements were carried out until the yellow ripening stage. Sampling was performed by hand always from the same rows of plots. *In situ* plant measurements were carried out before and after microalgae treatments. The following parameters were recorded: plant height, plate diameter, plate weight, thousand achene weight, yield (kg/ha), oil content, oil yield (kg/ha) and fatty acid composition. The effect of the microalgae treatments were evaluated by analysis of variance (ANOVA) and correlation analysis. Laboratory measurements were carried out at the Institute of Plant Biology, Faculty of Agriculture and Food Sciences, University of West Hungary, Mosonmagyaróvár.

In the experimental year 2013 the plants grew under extremely dry conditions during the growing season. Therefore crop safety and input efficiency become very important under such conditions. The treated and untreated (control) plants did not differ significantly regarding plant height. However, plate diameter, plate size, achene weight, oil content, oil yield and fatty acid composition (nervonic acid, linoleic acid, stearic acid, palmitic acid) differed significantly on plots treated by different microalgae compared to the control. Larger plate diameters resulted in bigger achene weight, which indicated a positive correlation between the two parameters. Crop yields were significantly increased in our experiment. Plants treated with 0,1% microalgal biomass of the strains MACC-612 *Nostoc entophyllum* and MACC-430 *Tetracystis* sp. had 14% and 12,2% yield increase compared with the control (3572 kg/ha), respectively. Oil content was significantly different on plots treated by different microalgae compared to the control (47,1%). Plots treated with the strains MACC-612 *Nostoc entophyllum* indicated the highest oil content value (49,4%).

The results proved that plant treatment with 0.1% suspensions of both microalgae positively affected the growth, development and consequently the yield of sunflower. Based on our current results and previous results performed on different

plant species, it can be stated that plant treatment with microalgae strains may be a good tool for increasing plant yield under stress conditions.

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# EFFECT ON THE GROWTH, CONDITION, NUMBER OF QUALITY AND OTHER PARAMETERS OF PEPPER KALDOM BY USING NOSTOC ENTHOPHYTUM MICROALGAE-TREATMENT

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The fact that sustainable development and environmental management comes to the front entails the change of agricultural production. The acceleration of global warming, and its conclusion – the extremely changeable weather (eg. drought, high temperature) are becoming major challenge for plant breeders. Several experiments demonstrate the possibility that plants are treated with microalgae are less impacted with environmental stress. The aim of our experiments was to develop a new cropping/production technology that could facilitate the production of red pepper in areas where no or very small amounts of irrigation water is available. The experiments were started in 2009 near Mőzs (Tolna county). The subject of the experiment was the *Capsicum annum* var. *Longum* Kaldóm species which had been treated with *Nostoc enthophytum* (MACC-612) cyanobacterial biomass that was from the Mosonmagyaróvár Algalculture Collection (MACC). The first treatment took place when the plants had 10 to 15 leaves, the second was when the plants had green buds, and the third treatment was carried out when the fruits first appeared. The plant populations of the experimental plots were treated regularly with MACC-612 algae biomass with 120, 120, 210 g/ha (0.3% concentration), or with 400, 400, 700 g/ha (1% concentration). The total volume of the spray was always 400, 400, 700 l/ha. During the experiments 7 parameters were examined: the number and length of the side branches were measured three times as well as the height of the plants. The measurements were done before the treatments in each case.

The average number of fruits was only measured at the third treatment. The direct bollworm infection was measured right before the harvest, while the total and the marketable yield was measured on the day of the harvest. Representative samples for the laboratory examinations were collected on September 6, 2009. The average number of seeds, and the average seed weight per crop was examined at the Institute of Plant Biology in Mosonmagyaróvár of the University of West Hungary. The number of vessels, the boll size, the pericarp thickness, the average sugar content and the weight of thousand seeds were determined. The results showed

that the parcels treated with the 1% concentrate suspension of *Nostoc enthophytum* (MACC-612) cyanobacterial biomass led to 20% more fruits and 29% more marketable yield. Compared to the control parcels the amount of side branches increased significantly (29%), as well as the average number of fruits (20%), and the number of mature leaves (18%). Concluding the examination of fruits, the same concentration of MACC-612 increased the pericarp thickness (28%), as well as the average weight of seeds (22%) the average boll size (15%) and the average fruit size (13%), too. It also increased the average weight of thousand seeds (12%), the average seed number of each fruit (9%) and the number of vessels in each pepper (7%). The direct bollworm-infection *Helicoverpa armigera* decreased by 41%. The treatments that were carried out with 0.3% concentrate had only been effective in some parameters.

In conclusion our data show, that the treatment of pepper with *Nostoc enthophytum* (MACC-612) cyanobacterial biomass in a concentrate of 1% had a positive effect on the growth, and condition of pepper and it has improved a number of quality and other parameters. The results of the experiment, as well as other experiments done before on pepper demonstrated the positive effect of the strain. This is why the registration of MACC-612 *Nostoc enthophytum*, and its involvement into cultivation on pepper is recommended.



# POSTERS

# THE PLOIDY AND GENOME COMPOSITION OF *SOLANUM TUBEROSUM* AND *SOLANUM BULBOCASTANUM* SOMATIC HYBRIDS AND THEIR DERIVED BACKCROSS PROGENIES

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Potato (*Solanum tuberosum*) as the third most important crop in the world, is essential component of the diet of humans and animals. The late blight of potato caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is recognized world-wide as the most devastating disease of potatoes that cause 16% annual yield losses. This oomycete has a capacity to develop resistance to modern fungicides, varies genetically for virulence and are able to overcome previously resistant potatoes.

For this reason many breeding programs are aiming to create resistant cultivars, using wild potato species which are resistant to this pathogen. *Solanum bulbocastanum* is a tuber-bearing wild species that shows durable race-non-specific resistance to late blight. The problem for traditional breeders is that this wild species is sexually incompatible with potato, because of differences in EBN (endosperm balance number). To overcome this problem somatic hybridization was used. Somatic hybrids (SH) were produced by protoplast electrofusion, using the cultivated potato and one accession of *Solanum bulbocastanum* carrying two resistance genes for late blight: *Rpi-blb1* and *Rpi-blb3*. The backcross generations were obtained by crossing the hybrid to another cultivar of potato. It is well known that an intracellular phenomenon, somatic incompatibility, can result in chromosome loss during hybrid plant regeneration or after backcrossing. Therefore it is important to re-analyze the hybrids and backcross progenies ploidy. One other important cytogenetic method is the multicolor genomic *in situ* hybridization (mcGISH), with which the genomic composition of somatic hybrids and backcross progenies can be seen.

The aim of our study was to determine the ploidy using flow cytometry and genome composition by mcGISH of three different somatic hybrids and their backcross progenies: BC1 and BC2, between potato + *S. bulbocastanum*.

Determination of ploidy level by flow cytometry (FC): The first leaf pairs of 8 weeks old *in vitro* plants were chopped with a razor blade in 800 µL of LB buffer. The leaf tissues in 800 µL of buffer were filtered over a 25-µm mesh and supplemented with 12.5 µL of 1 µg/mg propidium iodide (PI). The nuclear DNA content distribution was

analyzed with a Becton Dickinson FacScan flow cytometer and the data were analyzed using CellQuest software.

McGISH was performed in order to visualize simultaneously the chromosome of *Solanum bulbocastanum* ( $2n=2x=28$ , AbAb) and *Solanum tuberosum* ( $2n=4x=48$ , AAAA). Total DNA from *Solanum bulbocastanum* and *Solanum tuberosum* was labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) using the random primed labelling protocol. The hybridization mixture contained 60 ng each of the labelled probes/slide, dissolved in a 15  $\mu$ l mixture of 100% formamide, 20xSSC and 10% dextran-sulfate at a ratio of 5:1:4. In order to denature the probe DNAs, the hybridization mixture was heated to 75 °C for 8 minutes and then immediately cooled in ice. The chromosome spreads were denatured at 80°C in a high formamide concentration mixture containing 100% formamide, 20xSSC and 10% dextran-sulfate at a ratio of 15:3:3. Hybridization was performed at 42°C overnight. Streptavidin-FITC (Roche) and Anti-Digoxigenin-Rhodamine (Roche) dissolved in TNB were used in the detection phase. The last step was counterstaining with 2  $\mu$ g/mL 4,6-diamidino-2-phenylindole (DAPI) in an antifading solution (Vectashield, Vector Laboratories). The slides were screened using a Zeiss Axioskop-2 fluorescence microscope equipped with filter sets appropriate for DAPI, FITC, Rhodamine, and for the simultaneous detection of FITC and Rhodamin. Images were captured with a Spot CCD camera (Diagnostic Instruments) and processed with Image Pro Plus software (Media Cybernetics).

The results of FC determination of the somatic hybrids and BC progenies indicate that the ploidy of somatic hybrid plants remained 5x-6x, since the backcross progenies vary between 4x-5x. FC gives only an idea about the number of chromosomes but does not allow evaluation of parental chromosome contribution to the genome of the somatic hybrids and their progenies therefore GISH has to be investigated.

Repeated attempts of mcGISH become successful. The parental species have been also assessed as controls. Two pairs of *Solanum bulbocastanum* chromosomes gave stronger hybridization signals than the other chromosomes.

In total three hybrids, two BC1 and two BC2 progenies were analyzed. The chromosome constitution and genome composition could be revealed using mcGISH in the somatic hybrids and backcross progenies. In the hybrids the parental chromosomes were detected by differential fluorescent labelling. In the case of BC1 and BC2 progenies a decrease in the number of *Solanum bulbocastanum* chromosomes could be revealed. In several cases translocation between the chromosomes of the parental species was also observed.

Results indicate that the mcGISH is a valuable tool that can be used to successfully reveal the genome composition and genome dosage of the somatic hybrids and in the backcross progenies of potato + *S. bulbocastanum* hybrids.

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# VARIAMETRIC ANALYSIS OF EINKORN (*TRITICUM M. MONOCOCCUM*) SEED POPULATIONS: MEASURING GENETIC DISTANCE USING PHENOVARIATION

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Nine einkorn (*Triticum m. monococcum*) cultivars and landraces were studied by means of digital image analysis (Fovea Pro 4.0) for cultivar identification. DA (Discriminant Analysis), FA (Factor Analysis) and CA (Cluster Analyses) revealed that each einkorn varieties separated into distinct groups. These results potentially provide a digital tool for variety registration (DUS).

Einkorn (*Triticum monococcum* ssp. *monococcum*), one of the ancient diploid ( $2n=14$ ) *Triticums* (Table 1), was introduced into the Carpathian Basin by Neolithic tribes. Today, its cultivation is minimal, grown mostly in small, isolated mountain regions. However, einkorn is undergoing a renaissance with recultivation of old landraces due to its value in human diets. Here we applied Computer Assisted Seed Morphometry analysis of digital images of seed populations of nine einkorn varieties and landraces (200 seeds each) with a goal to provide new digital tools for variety registration.

Digital photos (pdf) were taken by Canon scanner, and pdf files were processed using the *Fovea Pro 4.0* computer program ([www.reindeergraphics.com](http://www.reindeergraphics.com)) in conjunction with Adobe Photoshop. Backgrounds of photos were processed first by 'magic wand', selected, cleared and edited to create black and white bitmaps. This was followed by 'remove scratches and dust', and finally, bright light reflection spots on seeds were removed by use of a 'fill holes' function. In total 33 seed size and shape parameters were measured: For Discriminant- and Factor Analysis the SPSS program package was used. For XY plots and Histogram analyses Microsoft Excel program was used. Of the 33 seed parameters, 17 were assessed by discriminant analysis (Area, Convex Area, Length, Breadth, Equiv. Diam., Inscib.Rad., Circum.Rad., Perimeter, Convex Perim., FormFactor, Roundness, Aspect Ratio, Solidity, Convexity, Symmetry, Radius Ratio, Elongation).

Based on these characters, cluster analysis (by SPSS program package) revealed that einkorn variety 'Schiemann' (Morocco) and 'Janicsár' show the closest seed morphological relationship. Landraces of 'Mv Alkor', 'Fazekas' and 'Agostyáni' grouped in a second cluster. A third group was comprised of three further landraces from the Carpathian Basin: 'Clusius', 'Pataki', and 'Gyulai'. The registered cultivar,

'Mv Menket' (Martonvásár), separated in a sole cluster due probably to the semi dwarf character of this variety. Two old einkorn landraces from the Carpathian Basin, 'Clusius' and 'Pataki' have the biggest seed size compared to 'Mv. Menket', a registered variety of Martonvásár, H, with the smallest size.

Common name	Genome(s)	Latin name	Traditional name
<i>(I.) Diploid (2x), Wild, Hulled</i>			
Wild einkorn	A <sup>m</sup>	<i>T. monococcum ssp. aegilopoides</i>	<i>T. boeoticum</i>
	A <sup>u</sup>	<i>T. urartu</i>	<i>T. urartu</i>
<i>Diploid (2x), Domesticated, Hulled</i>			
Einkorn	A <sup>m</sup>	<i>T. monococcum ssp. monococcum</i>	<i>T. monococcum</i>
<i>(II.) Tetraploid (4x), Wild, Hulled</i>			
Wild emmer	BA <sup>u</sup>	<i>T. turgidum ssp. dicoccoides</i>	<i>T. dicoccoides</i>
<i>Tetraploid (4x), Domesticated, Hulled</i>			
Emmer	BA <sup>u</sup>	<i>T. turgidum ssp. dicoccum</i>	<i>T. dicoccum</i>
	BA <sup>u</sup>	<i>T. ispahanicum</i>	<i>T. ispahanicum</i>
	BA <sup>u</sup>	<i>T. turgidum ssp. paleocolchicum</i>	<i>T. karamyshevii</i>
<i>Tetraploid (4x), Domesticated, Free-threshing</i>			
Durum (macaroni) wheat	BA <sup>u</sup>	<i>T. turgidum ssp. durum</i>	<i>T. durum</i>
Rivet (cone or English) wheat	BA <sup>u</sup>	<i>T. turgidum ssp. turgidum</i>	<i>T. turgidum</i>
Polish wheat	BA <sup>u</sup>	<i>T. turgidum ssp. polonicum</i>	<i>T. polonicum</i>
Khorasan wheat	BA <sup>u</sup>	<i>T. turgidum ssp. turanicum</i>	<i>T. turanicum</i>
Persian wheat	BA <sup>u</sup>	<i>T. turgidum ssp. carthlicum</i>	<i>T. carthlicum</i>
<i>Tetraploid (4x) - timopheevi group Wild, Hulled</i>			
	GA <sup>m</sup>	<i>T. timopheevii ssp. armeniacum</i>	<i>T. araraticum</i>
<i>Domesticated, Hulled</i>			
	GA <sup>m</sup>	<i>T. timopheevii ssp. timopheevii</i>	<i>T. timopheevii</i>
<i>(III.) Hexaploid (6x), Domesticated, Hulled</i>			
Spelt wheat	BA <sup>u</sup> D	<i>T. aestivum ssp. spelta</i>	<i>T. spelta</i>
	BA <sup>u</sup> D	<i>T. aestivum ssp. macha</i>	<i>T. macha</i>
	BA <sup>u</sup> D	<i>T. vavilovii</i>	<i>T. vavilovii</i>
<i>Hexaploid (6x), Domesticated, Free-threshing</i>			
Common (bread) wheat	BA <sup>u</sup> D	<i>T. aestivum ssp. aestivum</i>	<i>T. aestivum</i>
Club wheat	BA <sup>u</sup> D	<i>T. aestivum ssp. compactum</i>	<i>T. compactum</i>
Indian dwarf wheat	BA <sup>u</sup> D	<i>T. aestivum ssp. sphaerococcum</i>	<i>T. sphaerococcum</i>

Table 1. Wheat (*Triticum*) Taxonomy

Comparative yield tests were also conducted measuring the vigor of plant development, weed control characteristics, and the effects of soil quality on the yield. During diseases and pest control studies, leaf beetle (*Lema melanopa*) and grain beetle (*Anisoplia austriaca*) were detected. The best varieties were provided to plant breeders for further studies, and suggestions were given towards a digital registration system based on einkorn seed morphometry. The results may provide *new digital tools* for variety registration (DUS).

## ACKNOWLEDGEMENT

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# PRODUCING WHEAT/BARLEY INTROGRESSION LINES USING THE 2C GAMETOCID SYSTEM

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The 'Asakaze komugi'/'Manas' wheat/barley 4H disomic addition line have been crossed with the 'Chinese Spring'/'*Aegilops cylindrica* 2C addition line and the progenies were examined with fluorescence *in situ* hybridization (GISH, FISH) in order to identify chromosome rearrangements.

The barley chromosomes were detected by genomic *in situ* hybridization (GISH) using labelled total genomic barley DNA. In order to visualize the 2C chromosome of the *Aegilops cylindrica* Host ( $2n = 4x = 28$ , D<sup>c</sup>D<sup>c</sup>C<sup>c</sup>C<sup>c</sup>), C<sup>c</sup> genome donor *Aegilops caudata* L. ( $2n = 2x = 14$ , CC) genomic DNA was used as labelled probe. The 4HS-4AL and 4HS-4BL centric fusions were detected by means of fluorescence *in situ* hybridization (FISH) using repetitive DNA probes (Afa family, pSc119.2, pTa71).

Genetic diversity of wheat can be increased by interspecific and intergeneric hybridization in the tribe Triticeae. The intergeneric hybridization of wheat and barley makes it possible to transfer agronomically useful genes (e.g., drought tolerance, salt tolerance, earliness, and nutritional parameters) from barley into wheat.

Two sets of novel wheat/barley disomic addition lines were developed with agronomically adaptable winter barley cultivars in Martonvásár, the Mv9kr1/Igri (German two-rowed barley; 2H, 3H, 4H, 6HS, 7H and 1HS isochromosome) and the Asakaze komugi/Manas (Ukrainian six-rowed barley; 2H, 3H, 4H, 6H, 7H) additions. The Ukrainian six-rowed barley Manas has many useful agronomic characters, is well adapted to Central European conditions and has good winter hardiness, yield ability, drought tolerance and good tolerance of abiotic stresses such as Al and high NaCl concentration. Addition lines are the starting material for incorporating small segments of barley chromosomes carrying genes responsible for agronomically useful traits into the wheat genome, i.e. for producing translocation lines.

Some alien chromosomes called the gametocidal (Gc) chromosomes can induce chromosomal breakage resulting in the generation of deletions and translocations. Wheat/barley translocations will be induced by the gametocidal system using the 2C gametocidal chromosome derived from *Ae. cylindrica*.

The 'Asakaze komugi'/'Manas' wheat/barley 4H disomic addition line have been crossed with the 'Chinese Spring'/'*Aegilops cylindrica* 2C addition line and the progenies were examined with fluorescence *in situ* hybridization (GISH, FISH) in order to identify chromosome rearrangements.

Total barley genomic DNA was labelled with digoxigenin-11-dUTP. In order to visualize the 2C chromosome of the *Ae. cylindrica* C<sup>c</sup> genome donor *Aegilops caudata* L.

( $2n = 2x = 14$ , CC) genomic DNA was labelled with biotin-16-dUTP by nick translation and used as a probe. For GISH, 50-50 ng labelled barley and *Ae. caudata* total genomic DNA and 35x blocking wheat genomic DNA were used.

Digoxigenin-labelled DNA was detected with antidigoxigenin – rhodamine and biotin-labelled DNA was detected with streptavidin – FITC. The slides were counterstained with 2 µg/mL DAPI (4'-6-diamino-2-phenylindole) in Vectashield.

The 4HS-4AL and 4HS-4BL centric fusions were detected by means of fluorescence *in situ* hybridization (FISH) using repetitive DNA probes (Afa family, pSc119.2, pTa71).

Mitotic cells were examined with a Zeiss Axioskop-2 fluorescence microscope equipped with filter sets appropriate for DAPI (Zeiss filter set 1) and rhodamine (Zeiss filter set 15).

The Asakaze komugi/Manas 4H addition line was crossed with the CS/*Ae. cylindrica* 2C addition line to induce chromosome rearrangements between wheat and barley and F<sub>1</sub> seeds were produced. The expected genome composition of the F<sub>1</sub> seeds is 21<sup>II</sup> wheat + 1<sup>I</sup> barley + 1<sup>I</sup> 2C *Ae. cylindrica*. The F<sub>1</sub> seeds were selfed and analysed by GISH.

From 50 analysed plants we found 19 plants with 21<sup>II</sup> wheat + 1<sup>I</sup> barley + 1<sup>I</sup> 2C *Ae. cylindrica* genome composition and 6 plants which carried translocations.

The F<sub>1</sub> hybrids were backcrossed with the 4H wheat/barley addition line to obtain BC<sub>1</sub> plants. The expected genome composition of the progenies is 21<sup>II</sup> wheat + 1<sup>II</sup> barley + 1<sup>I</sup> 2C *Ae. cylindrica*. We found 18 plants with the expected genome composition out of 168 seeds.

BC<sub>1</sub> plants will be selfed and BC<sub>1</sub>F<sub>2</sub> seeds will be identified where the 2C chromosome has been eliminated and wheat/barley translocations occur. Intergeneric rearrangements will be detected using genomic *in situ* hybridization (GISH) analysis and with the help of molecular markers.

## ACKNOWLEDGEMENT

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# BIOTECHNOLOGICAL APPROACH TO CONTROL PARASITIC WEEDS

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Plant genera *Orobanche* and *Phelipanche* include root parasitic species. *Orobanche crenata*, *O. cumana*, *Phelipanche aegyptiaca* and *P. ramosa* attack agricultural important crops and negatively influence sensory quality of their fruits and yield. *P. aegyptiaca* and *P. ramosa* (L.) Pomel are widespread species, which cause huge damage on solanaceous crop, cruciferous plants, leguminous plants, sunflower or carrot in Middle East, south-western part of Europe, in India and South America.

Species of the genus *Phelipanche* have a special germination requirement by which they differ from other plants. Seeds have to undergo a moist and warm stratification before germination and then they need to be exposed to the germination stimulant(s) produced by host plants to be able to germinate. Among several classes of germination stimulants, the strigolactones (SLs) are the most studied compounds. The strigolactones are derived from carotenoid pathway (Matusova *et al.*, 2005), they influence different biological processes in plants as well as interaction of plants with arbuscular mycorrhizal fungi. Therefore SLs are characterized as a new class of plant hormones (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). The parasitic *Orobanche* and *Phelipanche* spp. are holoparasites, and the germinated seeds must attach to the roots of host plants within a few days to acquire all nutrients and water to grow and finish their life cycle. Biology of these root parasites is still poorly understood and for that reason development of efficient control strategy is not optimal yet. Several strategies including sanitation, solarization, nutrient composition of soil, use of herbicides, trap- and catch- crops or hand pulling are currently used to reduce the seedbank of parasitic plants in soil, but no one is really efficient in fight with these parasites. Nowadays promising approach to reduce these weeds from environment are biotechnological methods focusing on development of resistant crops. Introduction of parasitic plants *P. aegyptiaca* and *P. ramosa* into laboratory conditions and their use of *in vitro* cultures is another tool to study parasitic weeds in general, and genes involved in the host-parasite interactions in particular.

We established *in vitro* cultures of *P. ramosa*. The process includes preconditioning of seeds, germination and cultivation on solid or liquid nutrient solutions. Sterile seeds were preconditioned at 22 °C in a moist and dark environment for 12 days on glass fibre filter paper discs. During this period seeds released dormancy and became responsive to external germination stimuli. To induce germination, we used synthetic analogue of strigolactones GR24. Sufficient concentration of GR24 for induction of germination was 0,001 mg.l<sup>-1</sup>. *P. ramosa* seeds exposed to GR24 started to germinate after 3 days at 25°C in darkness. Germinated seeds with forming roots were transferred to B5 medium (Gamborg *et al.*, 1968) enriched with plant

agar, enzymatic casein hydrolysate, coconut water, sucrose and growth hormones IAA and GA<sub>3</sub>. Gradually during the cultivation at 25°C in darkness the germinated seeds turned into amorphous mass of cells, called calli. The morphology of calli was dependent on different seed origin. Most of the developed calli were able to infect roots of host plants in *in vitro* conditions and develop shoots.

## ACKNOWLEDGEMENT

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# MOLECULAR CHARACTERIZATION OF NOSTOC AND ANABAENA MICROALGAE ISOLATES

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The Algal Culture Collection of Mosonmagyaróvár (MACC) contains about more than one thousand strains, which is unique in Hungary and the fifth largest collection in Europe. Algae can be used for food and feed, as biopesticides, biofertilizers, for biomass and at last but not least for biofuel. The characterization of the MACC strains was primarily carried out using morphological markers, however, the identification was limited mostly to genera-level due to the small size and slight variety of the cells and the degradation of phenotypical characteristics during the breeding. Our main task is to develop a molecular taxonomy method, which enables a genotype level identification and the exploration of the strains' phylogenetic relationships, hereby facilitate the selection of strains for special economic utilisation.

28 deposits of *Nostoc* and 12 deposits of *Anabaena* microalgae isolate was analysed in this study. The 16S ribosomal RNA housekeeping gene and the gene *rbcLX*, which plays an essential role in CO<sub>2</sub> fixation were selected for the analysis. The primer combinations were designed based on published direct sequencing data. After the extractions of high quality DNA the specific fragments were amplified by PCR followed by sequencing of the purified products. Sequences were analysed using BLAST algorithm and the evolutionary relationships were determined using alignment and UPGMA algorithm based phylogenic tree.

Based on the *Nostoc* 16S rRNA gene sequences the tree presented in Figure 1 was built. Clusters were defined with over 80 % bootstrap threshold. Sequences were analysed using BLAST algorithm. A large number of highly similar non-*Nostoc* cyanobacteria sequences, highlighted in the red circled area, were found among the MACC *Nostoc* strains of. Furthermore, MACC-112 and MACC-210 as well as MACC-71 and MACC-139 strains seemed to be identical based on the similarity matrix values. Although *rbcLX* sequences were successfully recovered only for 15 strains, the analysis of these sequences provided similar results to 16S rRNA tree.

12 strains of *Anabaena* were examined. Based on the analysis of their 16S rRNA gene sequences MACC-57 and MACC-797, MACC-174 and MACC-260 as well as MACC-121, MACC-124, MACC-136 and MACC-238 strains resulted in identical similarity matrix values. Based on the BLAST analysis all the 12 strains belonged to the *Anabaena* genus.

The study carried out by the analysis of the 16S rDNA and *rbcLX* genes of *Nostoc* strains resulted some controversial sequences, based on which some of the analysed



# GENOTYPE IDENTIFICATION OF ANCIENT VINEGRAPE (*VITIS V. VINIFERA*) SEED REMAINS (15<sup>TH</sup> CENT. HUNGARY) BY DIGITAL MORPHOMETRY

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Digital image analysis of three ancient vinegrape seed remains, excavated from the 15<sup>th</sup> cent., and those of ten old grapevine varieties (200 seed each) were studied (33 seed parameters) by digital image analysis (*Fovea Pro4.0 program*) for genotype reconstruction of the ancient *Vitis* cultivars. Discriminant Analysis, Cluster, XY plots and histogram analyses revealed that seeds of two archaeological samples (11<sup>th</sup> and 13<sup>th</sup> sample) show the closest similarity to a currently grown old vinegrape *V.v.v.* 'Mézesfehér' (6<sup>th</sup> sample). Histogram analysis of seed parameter *Equiv. Diam.*(cm) of the archaeological sample *V.v.v.* 'Buda' (11<sup>th</sup> sample) showed diverse multimodal distribution compared to the unimodal distribution of 'Mézesfehér' (6<sup>th</sup> sample), which results indicate that the presumed ancestor old vinegrape cultivar 'Mézesfehér' went through a selection through the last five centuries, which narrowed the morphological diversity of this seed character.

Species of *Vitaceae* plant family are woody climbers comprising of 17 genera (*Acareosperma*, *Ampelocissus*, *Ampelopsis*, *Cayratia*, *Cissus*, *Clematicissus*, *Cyphostemma*, *Leea*, *Muscadinia*, *Nothocissus*, *Parthenocissus*, *Pterisanthes*, *Pterocissus*, *Rhoicissus*, *Tetrastigma*, *Yua* and *Vitis*), with a total species number of 700. Most genera of *Vitaceae* have  $2n = 38$  chromosomes ( $n = 19$ ), however species of *Muscadinia*, *Ampelocissus*, *Parthenocissus*, and *Ampelopsis* have  $2n = 40$  chromosomes ( $n = 20$ ), and species of the genus *Cissus* has  $2n = 24$  chromosomes ( $n = 12$ ).

Genus *Vitis* consists of about sixty inter-fertile species, including about fifteen species of agronomic importance. Of them, grapevine (*Vitis vinifera*;  $2n = 4x = 38$ ) has the most significant role. Genetically, vinegrape has a relatively small nuclear (nDNA) genome size ( $0.475 - 0.5 \times 10^9$  DNA bp), and regular sizes of cpDNA (160,928 bp, NCBI #DQ424856), and mtDNA (773,279 bp; NCBI #FM179380).

By today, thousands of grapevine varieties and sorts have been developed, which are generally classified in groups according to the final consumptions of wine grapes, table grapes, seedless grapes, and raisins. There have been great efforts to find morphological and molecular markers to determine and discriminate vinegrape varieties.

One of the morphological markers is based on seed morphology. Morphologically, it is easy to discriminate seeds of wild grape (*V. v. sylvestris*) from those of grapevine (*V. v. vinifera*) due to the lack of 'seed stalk' of wild grape seeds. However, it is very difficult to discriminate seeds of vinegrape varieties based on the morphological characters, particularly by the pair of ventral *infolds* and a dorsal *chalazal scar* of the seeds.

Here we aimed to discriminate, and identify three ancient grapevine specimens excavated from the Middle Ages (14<sup>th</sup> cent.), Hungary, by digital seed morphometry. For control, we used visually preselected seed populations of ten currently grown old grapevine cultivars from Hungary.

Digital photos of the ten old current vinegrape (*Vitis v. vinifera*) varieties (200 seeds each) and the archaeological specimens were taken by Canon scanner and, analyzed by Fovea Pro 4.0 program. Data of the 33 parameters were forwarded for Cluster- and Discriminant Analysis (SPSS program package).

In total, 33 seed parameters were measured, of them only eleven were grouped in the first and second functions of discriminate analysis: Function 1: Circum, Rad (cm), Length (cm), Perimeter (cm), External Perim (cm), Formfactor, Convex Perim (cm), Skeleton Length (cm), Function 2: Equiv. Diam (cm), Filled Area (cm<sup>2</sup>), Area (cm<sup>2</sup>), and Convex Area (cm<sup>2</sup>). It revealed that archaeological sample 11<sup>th</sup> ('Buda') and 13<sup>th</sup> ('Debrecen') showed the closest distances to currently grown old grapevine 'Mézesehér's (6<sup>th</sup> sample). This result may indicate the same origin and genotype of these archaeological samples. By accepting this indication we assume that currently grown old grapevine cultivar 'Mézesehér' survived the *Phylloxera* (an insect pest) in Hungary, which devastated European vineyards in the 1880s, and this results can also indicate the importance of replantations of this old grapevine varieties.

In the histogram analysis the seed parameter Equiv. Diam. (cm) of the archaeological sample *V.v.v.* 'Buda' (11<sup>th</sup> sample) showed diverse multimodal distribution compared to the unimodal distribution of 'Mézesehér' (6<sup>th</sup> sample). These results indicate that *V.v.v.* 'Mézesehér' went through a selection through the last five centuries, which narrowed the diversity of this seed character. By using large sample populations (n = 200) of seeds, we are more likely to avoid small sample bias in seed morphometric data since individual seeds may represent different ripening/developmental stages and/or differences in size and shape according to their positions in the berry.

To conclude, our results indicate the power of the digital seed morphometry to identify and reconstructs ancient *Vitis* seed remains for archaeobotanical use. The methods applied also were found to scale the genetic speed of domestication of *Vitis* cultivars.

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# LIGHT QUALITY INFLUENCES THE EXPRESSION LEVEL OF SOME CBF GENES

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The seasonal and daily changes of temperature and light conditions are the most characteristic features of the prevailing environment. Winter cereals have to survive the winter period, and it is well studied that during cold hardening the primary factor is low temperature. The *CBF* genes, encoding transcription factors are induced by short cold exposure. As transcription factors, they regulate other genes, thus initiating processes that lead to the formation of frost tolerance.

Previous studies show that light conditions such as light intensity, day length and light quality also play important role in the cold acclimation process. Plants sense the light spectra through different photoreceptors. Blue and UV-A wavelengths are perceived by cryptochromes and phototropins, red and far-red wavelengths are perceived by phytochromes. The phytochromes exist in two forms, the inactive red absorbing (Pr) and the active far-red absorbing (Pfr) form. The Pr form is converted to the active Pfr form by exposure to R-light, and is converted back to Pr form by exposure to FR-light or through dark reversion.

Phytochromes regulate the expression of several downstream genes like the Phytochrome-Interacting Factors (PIF). PIFs directly bind to the phytochromes and in the same time also bind to the G-box motifs in the promoters of the regulated genes. In *Arabidopsis* the regulation of the *CBF* pathway is mediated by the PHYB photoreceptor and two Phytochrome Interacting Factors (PIF 4 and 7). Phytochromes are negative regulators of the *CBFs*. In *Arabidopsis* the sensing of the low red/far-red ratio in the daylight, which is typical at twilight, are also mediated by PHYB. In that case the focus is shifted on the inactive Pfr form, which causes higher *CBF* expression levels.

In cereals only few papers have described the relationship between changing light conditions and the transcript levels of *CBF*-regulon so here we show that monochromatic lights affect differently the expression level of the *CBF* genes. Furthermore, by modifying the light spectra from high R/FR ratio to low R/FR it is possible to induce the expression of the *CBF*-regulon and also to increase the freezing tolerance.

First of all the light responses of the *CBF* genes and *Cor14b* were tested at different temperatures on *Triticum monococcum* cv. G3116 under blue, red, far-red and white illumination or kept in dark at 22°C or 15°C. The expression level of several *CBFs* and a cold-inducible effector gene, *Cor14b* was detected by quantitative real-time PCR method. Our results prove that there is interaction between the *CBFs* and the light-quality in cereals as well.

Thereafter the frost tolerance of the leaves were tested on the winter barley 'Nure', the winter wheat 'Cheyenne' and the winter einkorn 'G3116'. Beside white light, additional red or far-red or blue light was given to the plants at 15°C and the membrane injury was investigated by electrolyte leakage measurement at different freezing temperatures. Expression levels of several target genes were also detected. Whole plants were frozen at the end of the treatments and regeneration ability, survival rates were also calculated. It was obviously proven that the far-red light treated plants showed higher frost tolerance and higher expression level of several frost-related genes.

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# SIGNIFICANTLY INCREASED GLUTATHIONE-S-TRANSFERASE ACTIVITY IN 35S (CaMV)-ZMGSTF4 TRANSGENIC *ARABIDOPSIS THALIANA*

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Plant GSTs (glutathione S-transferases) (EC 2.5.1.18) are a large and diverse stress-protective enzyme family, which catalyze the conjugation of the tripeptide glutathione (gamma-L-glutamyl-L-cysteinylglycine, GSH) with a wide variety of harmful electrophilic xenobiotics. *E.g.* plant resistance to chloroacetanilide herbicides is mainly caused by the functions of GSTs in maize and soybean, which, in case of atrazine, results in GSH-atrazine a complex. Recent results revealed that GSTs are also involved in heavy metal stress defense mechanisms in plants. Genes encoding for GSTs are grouped in diverse gene families of A, B, D, K, M, O, F, T, U, Z, L, M, S. In *Arabidopsis*, 51 *AtGST* isoenzyme genes were cloned belonging to F – Phi, T – Theta, U – Tau, Z – Zeta families. In maize 42 *ZmGSTs* (12 F, 28 T, and 2 Z), and in soybean 25 *GmGSTs* (20 T, 4 F, and 1 Z) are known.

Genetic engineering is a powerful tool to study plant metabolic pathways. Overexpression of specific genes helps to clarify their physiological roles in the metabolism grown under different stress conditions. Deeper understanding of the biochemical pathways contributing to the processes of uptake, translocation and accumulation of heavy metals, and tolerance of phytotoxic chemicals will greatly help the improvement of phytoremediation potential of plants. Glutathione S-transferases seem to be valuable targets for these purposes. Glutathione S-transferases are considered to play an important role in heavy metal stress, especially through detoxification.

The 35S(CaMV)-*ZmgstF4* transgenic *Arabidopsis thaliana* (ecotype: Col-5), (EMBL: U12679 / X79515; Uniprot: P46420), used in the study presented. The transgene is driven by cauliflower mosaic virus (CaMV) 35S promoter, using pCAMBIA1301 binary vector, and it was introduced by floral dip transformation method based on hygromycin phosphotransferase (*hpt*) gene as selectable marker. Here we report a study of GST enzyme activity of WT and 35S(CaMV)-*ZmgstF4* transgenic (TR) *Arabidopsis*.

Numerous plant species are able to tolerate toxic substances and heavy metals from polluted soils and air, such as the annual species *Thlaspi caerulescens*, a known nickel (Ni) and zinc (Zn) hyperaccumulator plant; *Brassica juncea*, a Pb accumulator; and the arsenic (As) hyperaccumulator *Pteris vittata*. Metallocrops such as oat (*Avena sativa*), barley (*Hordeum vulgare*) and Indian mustard (*B. juncea*) also tend to take up

high level of Cu, Cd and Zn in hydroponics. The level of these natural tolerances can be highly improved by genetic transformation

In the study presented Clones of 35S-*Zmgstf4* transgenic (TR) *Arabidopsis thaliana* expressing the *Zmgstf4* gene of *Zea mays*, was tested for stress-inductive GST (glutathione S-transferase) activity following treatments with the heavy metals Zn, Cd, the chloroacetanilide herbicide metolachlor (2000  $\mu$ M), and elevated concentration of  $(\text{NH}_4)_2\text{SO}_4$

WT plants treated with Zn and Cd showed typical heavy metal toxicity symptoms: loss of chlorophyll and leaf turgor. The symptoms of metolachlor (200  $\mu$ M) were also significant; apparently, transgenic plants were unaffected by metolachlor compared with WT plants, which died after 11 days. The root development of WT plants was also seriously inhibited in WT.

The GST activity of the TR plants 75% higher than in the WT plant. After Cd (20 and 30  $\mu$ M), but not at Zn (150 and 1500  $\mu$ M) exposure increased GST activities were measured in both WT and TR plants, but the induction in TR plants was significantly higher.

Our results has indicated that overexpression of GST enzymes can play important roles in the detoxification of heavy metals, and tolerance to herbicides. The overexpression of *Zmgstf4* gene was also found to increase resistance against chloroacetanilide herbicides.

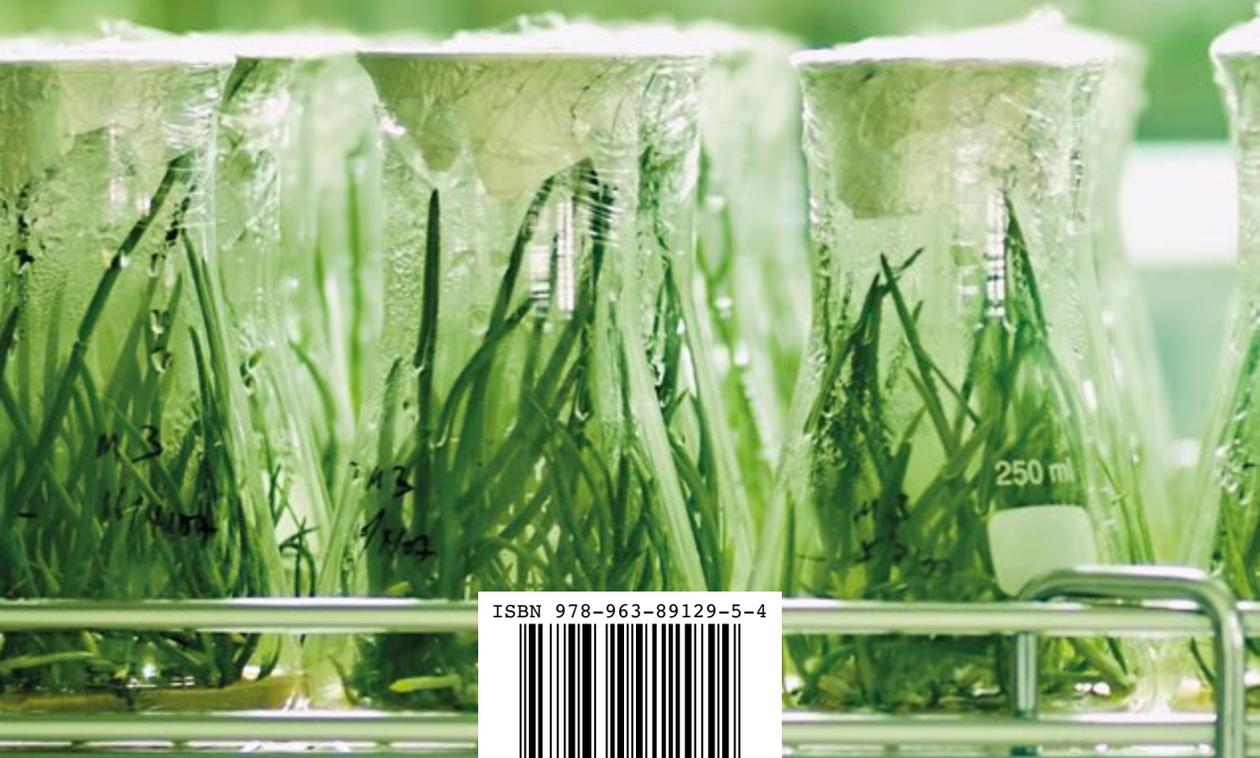
Ammonia is not a known inducer of plant glutathione S-transferases (GST), however several stress-factor can activate GST isoenzymes. The primary goal of this experiments was to show how the ammonium salts affects on GST enzyme activities The results shows that  $(\text{NH}_4)_2\text{SO}_4$  induce the GST activity even at low concentrations.

In conclusion, *Zmgstf4* transgenic *Arabidopsis* plant investigated in this study provided new data on the understanding of plant GST functions with indications in their use in phytoremediation. Results also show the applicability of the *Zmgstf4* gene in molecular plant breeding for phytoremediation purposes.

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