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EcoTILLING analysis of candidate genes for drought tolerance in barley

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The development of new barley varieties with improved drought tolerance is one of the main breeding objectives in Hungary, because drought is a main factor limiting the yield of cereals including barley. The development of stress-tolerant varieties with yield stability will help to reduce the risk in barley cultivation. The probability of a successful breeding for drought tolerance is largely dependent on the understanding and knowing of the genetical factors that regulate this highly quantitative trait.

In this project drought tolerance related candidate genes were analyzed by using the EcoTILLING (Comai et al. 2004) technology. EcoTILLING is a high throughput, low cost technique for rapid discovery of polymorphisms in natural populations. It is a variant of TILLING (Targeting Induced Local Lesions IN Genomes), (Colbert et al. 2001) is based on certain PCR steps, such as the formation of heteroduplexes and a nuclease cutting DNA mismatches. It allows both SNP discovery and haplotyping through the sequencing of unique haplotypes.

We have established the EcoTILLING technology in order to identify putative SNPs and small INDELs in a set of 96 barley cultivars and wild germplasm containing drought tolerant and sensitive genotypes (cultivars and landraces and wild relatives) collected worldwide. Target genes were selected based on studies dealing with drought tolerance. Candidate genes are dedicated as potentially involved in the variation of key agronomic traits. The identification/determination of natural genetic variation in candidate genes can provide valuable information about gene function.

In this pilot study 7 drought related barley candidate genes were screened. In the case of 4 genes overlapping amplicons were designed, trying to cover the whole gene in the genetic diversity screens. For these 4 genes also more easily detectable markers were created after the evaluation of the obtained haplotypes sequences allowing distinguishing the main haplotypes. In the case of 3 candidate genes only one primer pair was planned based on the available mRNA sequences.

EcoTILLING reactions were performed in one-well format using fluorescently labeled nucleotides and after heteroduplex formation ENDO-1 and Cel-1 treated products were visualized on an ABI PRISM 377 sequencer.

Untill now more than one hundred unique haplotypes identified for 9 genes (HvARH1, HvDREB1, HvDRF1, HVA1, HvNHX1, HVP1, HvPPD-H1, HvNUD and HvPRPX) in 18 EcoTILLING screens. It's including more than 1.5 million base pairs sequence. The number of haplotypes identified for screened amplicons ranged from 2 to 9. Overall, 185 single nucleotide polymorphisms and 46 insertions/deletions were finded with a mean of 1SNP/92 bp and 1INDEL/372 bp genomic sequence.

In four candidate genes (HvARH1, HVA1, HvDRF1, HvSRG6) a set of informative polymorphisms were converted into easily detectable genetic markers, which are useful for marker assisted selection.

The obtained sequence/haplotype information will be used for development of further easily detectable genetic markers (potential „within gene marker") useful for linkage mapping and Marker Assisted Selection. Functional alleles can be directly integrated in barley breeding programs for improvement of drought tolerance.

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Application of synthetic antisense oligodeoxynucleotides in higher plants

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Antisense oligonucleotides *i.e.* short, synthetic strands of DNA or analogs that are complementary to a target DNA or RNA along with short interfering RNAs (siRNAs), 21-25 bp dsRNA with dinucleotide 3' overhangs became a powerful tool for the functional genetics. These structures are designed to interfere with nucleic acid metabolism, most preferentially with transcription, translation or splicing. Sequence-selective inhibition of gene expression is applied extensively for elucidation of complex gene expression patterns or validation of results gained from high throughput genomic experiments such as DNA-arrays. Common and attracting features of both antisense oligo and siRNA are that they act in a dose-dependent reversible manner, while no genetic transformation is required.

Though the sequence-selective gene-silencing by these synthetic oligonucleotides is quite general phenomenon for all organisms, only few applications are described for plant systems. We elaborated several methods for the introduction of oligonucleotides into monocot and dicot plants. By fluorescent labeling, we examined the uptake efficiency and inner traffic of these molecules, and determined the optimal conditions of treatment.

To demonstrate the antisense inhibition, we chose phytoene desaturase (pds) as a model gene which is a key-enzyme of the carotenoid biosynthesis in Triticum aestivum and Nicotiana benthamiana. Selection of the antisense target sites was made by a multistep optimization process which raises the targeting efficiency significantly. By means of quantitative RT-PCR method, we demonstrated sequence-specific knock-down of pds mRNA level. We followed the phenotypical changes of the plants by chlorophyll fluorometry and carotenoid content measurement, thus significant loss PDS function was demonstrated. at a significant level. Our experiences open the way for applying the antisense oligonucleotide technique for elucidation of real genetic problems.

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Epigenetic changes in tumor-associated myofibroblast

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The tumor microenvironment is an important factor in cancer development and progression.

In the stroma of various epithelial tumors the predominant cell types are myofibroblasts. These spindle-shaped cells were originally described in skin wounds where they facilitate wound healing. Myofibroblasts are differenciated fibroblasts expressing specific markers like alpha smooth muscle actin, vimentin and secreting several extracellular matrix proteins (Desmoulière et al. 2004). Compared to normal tissue, the number of myofibroblasts is increased in the tumor stroma and the shape and distribution of the cells are altered as well. The observed morphological changes are believed to be partially due to epigenetic effects, which cause an altered gene expression profile without influencing the DNA sequence. The epigenetic changes occuring in tumor- associated myofibroblasts are poorly understood (Jiang et al. 2008).

In order to compare epigenetic characteristics and gene expression pattern of tumor-associated myofibroblasts with that of normal myofibroblasts in molecular level, we used primary myofibroblast cultures obtained from the gastrointestinal tract. Cells isolated from tumor stroma or from healthy tissues near the tumor margins were provided by Dr. Peter Hegyi (Dept. of Internal Medicine, Faculty of Medicine, University of Szeged). We studied epigenetic changes such as histone H3 and H4 acetylation and methylation in tumor-associated myofibroblasts by immunocytochemistry. The results indicated lower levels of histone H4 acetylated on lysine 8, 12, 16 and H3 dimethylated on lysine 9 in tumor-associated myofibroblast compared to normal cells. Semi quantitative determination of the level of particular histone modifications by immunoblots using modified histone specific antibodies supported and validated the observed epigenetic alterations. The expression profile of subunits of histone acetyltransferase (HAT) complexes were determined by quantitative RT-PCR. The analysis indicated that the mRNA levels corresponding to the *ada2a, ada3* and *gcn5* genes, which code subunits of several HAT complexes, such as SAGA and ATAC, were lower in tumor-associated samples, then in their wild type counterparts.

The role of myofibroblasts in cancer metastasis is also suspected (De Wever et al. 2008). They can secrete many proteolytic enzymes, which digest extracellular matrix in order to promote cancer cell invasion. Therefore we were interested in studying the expression, secretion and activity of the gelatinase enzymes, matrix metalloproteinase 2 and 9 (MMP-2, 9) in the myofibroblast cultures. Based on quantitative RT-PCR data we performed, we concluded that the expression of MMP-2 was elevated in tumor-associated myofibroblasts, while the messenger of MMP-9 was detectable neither in the tumor-associated nor the control cells. We have also performed a gelatin zymography to detect the activity of MMP-2 and 9. For this protein extracts from tumor associated and normal cells, and as well secreted protein samples obtained from the culture media were loaded onto polyacrylamide gels co-polymerized with gelatin and resolved under nondenaturing conditions. Development of the gels with protein specific stain indicated strong MMP-2 activities in both samples, while the tumor-associated myofibroblasts secreted more MMP-2 enzymes to the extracellular space.

In the forthcoming months we plan to further investigate the differences in the gene expression profile of tumor-associated versus control myofibroblast using microarray. We expect that the results will broaden and refine our understanding on the role of myofibroblasts in tumor formation and invasion.

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