**Plan of Work for the OSIRIS Project**

The overriding goals of the OSIRIS activity are the application of the CRISPR/Cas9 system for site-directed mutagenesis to knock out three genes that regulate spike architecture in wheat, thereby generating novel spike phenotypes with increased yield potential.

**Induced mutations by application of CRISPR/Cas9 on three spike architecture genes of wheat; mutant analyses by NGS amplicon sequencing and phenotypic assessment of homozygous mutant plants**

We have cloned the gene underlying spike-branching in 'Miracle-Wheat' and found that it is an ortholog of *com2* in barley (Poursarebani et al., 2015). Our two years of field experiments with six 'Miracle Wheat' accessions showed a significant increase in spike dry weight at anthesis, higher grain number per spike (+45%) and more grain yield per spike (+28%) as compared to canonical spike forms of six adapted elite durum wheat cultivars (Poursarebani et al., 2015). It is highly likely that the other two genes, i.e. *COM1* and *Vrs4* from barley, are similarly involved in branch repression in wheat. On this basis, we will apply RNA-guided endonucleases (RGENs, based on the bacterial CRISPR/Cas9 immune system) to knock-out the spike architecture genes *TaCOM1*, *TaBH*, and *TaVrs4* in hexaploid wheat (cv. Bobwhite; Appendix 2) to create and assess different putative mutant classes per target-gene; i.e. single (AA, BB, or DD single mutants), double (AA/BB, AA/DD, BB/DD double mutants), or triple (AA/BB/DD) mutants of the homoeo-alleles. Thus, in total seven genotypic classes per target-gene will be investigated phenotypically. Once $T_0$-plants will be obtained for individual target genes, plants will be sequenced using latest NGS methodology of the in-house Illumina HiSeqTM 2000 platform following a newly established protocol for amplicon sequencing (IPK, Genome Diversity Group, Dr. A. Himmelbach; personal communication). To detect mutations in the transgenic plants and their progeny, all three homozygous target-gene copies will be amplified and sequenced using homoeo-allele specific primer pairs. All PCR products per target-gene for each individual will be pooled and labelled with a specific barcode. Several post-amplification steps will be carried out per pool prior to the start of the HiSeq sequencing run. All steps follow the manufacture instructions except some modifications through which the IPK protocol was developed (IPK, Genome Diversity Group, Dr. A. Himmelbach; personal communication). Sequence assembly, quality control, haplotype phasing and disentangling reads belonging to the different homoeologous copies will be performed as previously described for amplicon sequencing in polyploid species (Brassac and Blattner, 2015). Populations of doubled haploids (DH) will be created that are entirely homozygous among which the desired genotypes (as specified above) can be readily identified. However, as a backup approach for maintaining mutant material and generating homozygous lines, the primary mutant plants ($T_0$ generation) will also be self-pollinated to obtain conventional $T_1$ and $T_2$ families. Selected homozygous single, double or triple mutants for each of the genes will be phenotypically evaluated (Appendix 2). Based upon our preliminary expression analyses of these genes in hexaploid wheat, we anticipate mutating at least two out of the three homoeo-alleles in order to obtain phenotypes. For initial analyses, plants will be phenotypically evaluated under two conditions (greenhouse and field-like conditions) to identify the most promising wheat mutants with improved sink capacity and yield potential. To this end, relevant spike- and yield-related characters will be scored to assess the performance of the corresponding mutants. The first character category includes spike-branching related traits such as, number of rachis nodes, number of spikelets per node, number of nodes per spike with supernumerary spikelet (SS), number of nodes with branch-like structures and number of nodes per spike with non-SS or non-branching as described elsewhere (Echeverry-Solarte et al., 2014). These traits will be measured on four spikes of primary tillers. The number of additional spikelets observed on these four spikes and control plants, including different levels of branching will be determined. Additional measures will also be considered, such as number of spikes per plant, spike length, spike dry weight, heading time, plant height, leaf area, and internode weight (last two or three below spike). For each trait, the mean from four samples will be calculated for analyses.

**Task1** (involved partners: **PI_1 and PI_2**): Design and construction of RGEN vectors, transformation and DH plant production.

**Task2** (involved partner: **PI_1**): Genotyping of $T_0$ and $T_1$-DH plants for mutation detection, creation of $T_1$ and $T_2$ plants, and their phenotypic evaluation in greenhouse and field-like conditions.

**Milestones** (34-36 months): Application of CRISPR/Cas9 genome engineering system on three spike architecture genes in hexaploid wheat, through which wheat genotypes with novel spike architecture and improved yield potential will be created.

**References**

Poursarebani N, et al. (2015) The genetic basis of composite spike form in barley and 'Miracle-Wheat'. Genetics 201:155-165

Echeverry-Solarte M, Kumar A, Kianian S, Mantovani EE, Simsek S, Alamri MS, Mergoum M (2014) Genome-wide genetic dissection of supernumerary spikelet and related traits in common wheat. Plant Genome 7:10.3835/plantgenome2014.3803.0013