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Determining molecular functions of the *Ant27* locus that controls proanthocyanidin synthesis in barley grain

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Motivation and aim: Proanthocyanidins (PAs) are oligomeric flavonoids belonging to plant polyphenols. These compounds accumulate in seed coat of barley grain, having an important role in dormancy maintaining and protection of embryo from severe environments. Besides that, they have a significant impact on grain quality determining its intended use. To study genetics control of PAs synthesis in barley more than 700 *anthocyanin-less* (*ant*) mutants grouped into 30 loci, have been obtained by mutagenesis. Molecular functions were determined only for 10 of them. The aim of this study was to determine localization in genome and molecular functions of the *Ant27* locus that control PAs synthesis in barley grain.

Materials and methods: Two PA-free mutants *ant27.488* and *ant27.2043* obtained by mutagenesis applied to cultivars Zenit and Arena, respectively, were get from NordGen (Alnarp, Sweden). F_2 population derived from the PA-free *ant27.488* mutant and wild type cultivar Quench counting for 699 hybrids was used for bulked segregant analysis (BSA). Soaking the grains in NaOH allowed phenotyping the hybrid plants for PAs in grain. Pooled DNA of the hybrids accumulating or absence PAs, together with DNA of the parental samples was genotyped by SNP-chip Illumina Infinium Barley 50K (TraitGenetics, Germany). The data on SNP-genotyping were analyzed by Flapjack v.1.21.02.04. In the identified region, Arabidopsis orthologous genes engaged in PAs biosynthesis were found using TAIR database. The full length nucleotide sequences of the candidate gene in the mutants and their parental cultivars were determined by Sanger sequencing. CAPS markers were developed based on the SNPs revealed between the mutants and corresponding parental cultivars and used for genotyping F_2 hybrids *ant27.488*xQuench to validate the candidate gene. The transcription of the candidate gene and a set of flavonoid biosynthesis structural genes was analyzed in developing grain by quantitative RT-PCR. To reveal phenotypic effects of the mutations the cellular architecture of the epidermis in roots of seedlings and adult pre-boot leaves, the germination rate and growth parameters were compared between the mutants and their parental cultivars. Transcriptomic analysis of developing grain of *ant27.488* and Zenit was carried out by NextSeq™ 550 RNA sequencing.

Results: By BSA the *Ant27* locus was prescribed to a region of 170 Mb of chromosome 5H, in which the *HvWRKY40* gene orthologous to Arabidopsis *Ttg2*, participating in regulation of the PAs synthesis in seed coat was identified. Besides PAs synthesis, this gene control trichome development and mucilage production in seeds coat of Arabidopsis. In barley, comparative transcription analysis of flavonoid biosynthesis structural genes showed decreased expression of *Chs*, *F3h*, *Dfr*, *Lcr*, and *Aha10* in seed envelopes of the both mutants in comparison with their parental cultivars. The data imply that the *Ant27* gene has regulatory functions in PAs synthesis confirming that it may be *HvWRKY40*. Sequencing the gene in both mutants revealed the following SNPs: a G/A mutation at position 570, which is located before the translation start site in *ant27.488*, and a C/T mutation at position 717, resulting in a change from glutamine to a stop codon in *ant27.2043*. The coincidence of the mutant allele *ant27.488* and the phenotype in the F_2 hybrid population of *ant27.488* x Quench, where all 10 PA-free hybrids among the 165 analyzed carry the mutant allele in a homozygous state and all 155 PA-positive hybrids were either homozygous or heterozygous for the wild-type allele, confirmed the *HvWRKY40* gene as *Ant27*. The mutations affect the germination rate and growth parameters of barley plants demonstrating the pleiotropic functions of the gene. Comparative transcriptomic analysis revealed 1937 down-regulated and 670 up-regulated genes in the *ant27.488* mutant compared to the parent *cv*. Zenit at the late milk stage of grain development, while at the early dough stage 1636 and 966 genes, respectively were revealed. The flavonoid biosynthesis genes were expressed differently at the late milk stage, which was identified as a crucial stage for the regulation of PAs synthesis. Besides flavonoid biosynthesis pathway, the genes associated with response to abscisic acid, hydrogen peroxide and salt stress were identified among down-regulated in the mutant. Among the up-regulated genes in the mutant, those associated with DNA repair processes and cytoskeletal reorganization were identified. This may reflect the activation of compensatory mechanisms in response to disruption of PAs synthesis, which are protective compounds.

Conclusions: The gene *HvWRKY40* was identified as a candidate for the *Ant27* locus mapped to chromosome 5H. This data serves as a basis for further identification of other genes involved in PA synthesis, as well as for breeding PA-free barley varieties.

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