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WHEAT KERNEL ALPHA-AMYLASE/TRYPSIN INHIBITOR (ATI) GENE SILENCING PROCEDURES CAUSE PLEIOTROPIC EFFECTS AND AFFECT ATI PRO-INFLAMMATORY ACTIVITY

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Wheat is one of the main crops used as staple food, but it can also trigger adverse allergic reactions as well as intestinal and extra-intestinal inflammatory diseases, such as celiac disease and non celiac wheat sensitivity (NCWS). a-Amylase/trypsin inhibitors (ATI) are central wheat allergens and activators of innate immunity via their binding to the CD14 MD2 TLR4 (toll like receptor 4) complex on myeloid cells. ATI are encoded by a multigene family dispersed over several chromosomes in wheat. Tetrameric ATI CM3 and CM16, and dimeric 0.28 are major allergens in baker's asthma, the most common occupational asthma.

With the aim of obtaining wheat plants with decreased expression of specific ATI proteins, to ascertain the role of these proteins in allergy and NCWS and to use them as a basis to develop wheat varieties that are safer for allergic and NCWS patients, we have recently produced bread wheat lines with *CM3*, *CM16* and *0.28 ATI* genes silenced by means of RNAi (RNAi lines), and durum wheat lines with *CM3* and *CM16 ATI* genes silenced by Genome Editing using CRISPR/Cas9 (GE lines).

Quantitative ATI extracts from flours from these RNAi and GE lines, along

with the corresponding wild type genotypes, were tested for their TLR4-stimulating bioactivity *in vitro* using a Hela TLR4 dual luciferase reporter cell line. These cell lines produce a dose dependent luminescence signal after lysis of the reporter cells. Potential LPS contaminations was removed by prior polymyxin B affinity chromatography.

RNAi lines resulted in increased TLR4-stimulating bioactivity compared to the wild type reference genotype (bread wheat cultivar Bobwhite). These lines show several pleiotropic effects, varying from non-specific silencing of high molecular weight glutenin subunits to overexpression of g- and w-gliadins and other proteins with trypsin inhibition activity.

Differently, GE lines showed a slight reduction of bioactivity in one line (R298c), while the other line (R598b) was comparable to the respective wild type (durum wheat Svevo). Seed protein patterns were as expected in GE lines, with absence of the two target proteins CM3 and CM16, but maintained gluten and soluble protein patterns. Thus, GE technique proved a powerful genome modification tool with no marked pleiotropic effects, and the *in vitro* bioactivity readouts identify a need for more targeted deletion of ATI other than CM3 and CM16 in wheat, to reduce TLR4 proinflammatory activity.