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ORIGINAL PAPER

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INFLUENCE OF CCC AND TRINEXAPAC-ETHYL ON THE EXPRESSION OF GENES INVOLVED IN GIBBERELLIC BIOSYNTHESIS AND METABOLISM PATHWAY IN ISOGENIC LINE WITH *Rht12* DWARFING GENE

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ABSTRACT

Occurrence of lodging is a result of environmental stress factors and causes significant losses in crop yields. One of the major factors determining plant resistance to lodging is stem length. Thought, the introduction of the dwarfing genes into genome or application of growth regulators, that inhibit gibberellins biosynthesis, are known as the most important approaches in lodging prevention. In this study we analyzed the influence of chlormequat chloride (CCC) and trinexapac-ethyl application on transcriptome of common wheat (*Triticum aestivum* L.) 'Bezostaya 1'. For analysis, the tall control line and isogenic line carrying *Rht12* dwarfing gene were selected. Subsequently, the real-time PCR technique was used to determine the expression of five genes encoding enzymes involved in gibberellins biosynthesis pathway (CPS, KS, GA20ox, GA3ox and GA2ox).

Key words: common wheat, lodging, plant growth regulators, *Rht12* dwarfing gene, gibberellins biosynthesis pathway

INTRODUCTION

One of the most important factor, which determine good and stable yield of crops is lodging resistance. The major strategy chosen by cereal breeders with the purpose of obtaining the lodging resistant wheat cultivars is based on introduction of dwarfing genes from Japanese wheats [Gale and Youssefian 1985, Worland and Law 1986, Worland et al. 1990, Börner et al. 1996; Kowalczyk and Miazga 1996, Kowalczyk 1997, Worland et al. 1998, Borojevic and Borojevic 2005, Lou et. al. 2016]. Dwarfing genes can be divided into insensitive or sensitive to exogenous gibberellic acid application [Chen et al. 2015]. In wheat, mainly GA-insensitive dwarfing genes have been introduced to short-straw cultivars. Analysis revealed that they reduce plant height and increase number of grains per spike [Gale and Youssefian 1985, Börner et al. 1993, Kowalczyk et al. 1997, Miazga et al 1997]. Within the GA-sensitive dwarfing genes, strong effect of plant height reduction was observed in cultivars with introduced *Rht12* gene [Konzak 1982, Gale and Youssefian 1985]. The chromosomal location of *Rht12* gene was determined by Sutka and Kovacs [1987] on 5A chromosome. Korzun et al. [1997] used of microsatellite markers and mapped *Rht12* to the distal part of chromosome 5AL, approximately 2.6 cM from locus *Xwms291* and

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identified an additional marker that is tightly linked to that gene.

Molecular analyzes revealed that some of the cereal dwarfing genes encode modified enzymes involved in the gibberellin biosynthesis pathway. Gibberellins (GAs) are plant hormones mainly responsible for the regulation of growth and development [Olszewski et al. 2002]. Bioactive gibberellins are synthesized from trans-geranylgeranyldiphosphate (GGDP) through a multi-step conversion catalyzed by seven different enzymes localized in the chloroplasts, the ER membrane and the cytoplasm [Yang et al. 2009].

Another method to prevent plant lodging is application of plant growth regulators [Olumekun 1996, Kulig et al. 2001, Rajala and Peltonen-Sainio 2001, 2002, Stachecki et al. 2004, Matysiak 2006]. To control lodging, they are applied at the beginning of stem elongation (e.g. CCC – chlormequat chloride), or at the flag leaf stage (e.g. ethephon). Application of the growth retardants cause inhibition of gibberellins (GA) biosynthesis [Rademacher 2000, Espindula et al. 2009]. It results in increasing root growth, or a higher root/shoot ratio under field conditions. Growth regulators modify tiller number, especially when applied as a seed treatment, or during early growth stages [Rajala and Peltonen-Sainio 2001]. Trinexapac-ethyl and chlormequat chloride, despite coming from various chemical groups, have a similar mechanism of activity. They inhibit the synthesis of the enzymes necessary for the biosynthesis of gibberellins in plants. Visual effect of trinexapac-ethyl and chlormequat chloride is the reduction in plant height, thus increasing the resistance to lodging. Therefore, both active substances are widely used in the agricultural practice [Rademacher 2000, Matysiak et al. 2010].

The aim of this paper was characterization of CCC and trinexapac-ethyl influence on transcripts induction in common wheat cv. Bezostaya 1 isogenic line with dwarfing gene in locus *Rht12*, using RT-PCR. Moreover, the expression of five genes encoding enzymes of the gibberellins biosynthesis pathway: CPS (encoding ent-copalyl diphosphate synthase), KS (encoding ent-kaurene synthase), GA20ox (encoding GA20-oxidase), GA3ox (encoding GA3-oxidase) and GA2ox (encoding GA2-oxidase) were analyzed using qPCR method. Expression of these genes was analyzed before and after plant growth regulators treatment.

MATERIALS AND METHODS

Plant material. Isogenic line of 'Bezostaya 1' common wheat cultivar with *Rht12* dwarfing gene and its respective control tall form 'Bezostaya 1' *rht* were used for experiments. Tested lines were kindly supplied by Tony Worland from John Innes Centre (Norwich, UK).

Kernels of the analyzed forms were surface sterilized using a gaseous chlorine for 5 hours. In the next step, sterile kernels were put onto moist filter paper in Petri dishes and placed in growth chamber at 25°C, 85% humidity and absence of light. After three days, the seedlings were transferred to cuvettes filled with perlite and placed in a growth chamber under a photoperiod of 16 h day / 8 h night and 24°C. After seven days, the seedlings were treated with growth regulators. The two formulations: Stabilan 460 SL and Moddus 250 EC were used in the experiment in concentration recommended for commercial use. These preparations contain different active ingredient: chlormequat chloride (CCC, 2-chloroethyl(trimethyl)azanium chloride) and trinexapac-ethyl (TE, ethyl 4-[cyclopropyl(hydroxy)methylidene]-3,5-dioxocyclohexane-1--carboxylate), accordingly. The concentrations of the growth retardants were as follows: 6.750 mg l^{-1} for CCC and 500 mg l^{-1} for TE. For RT-PCR analysis plant material was collected after 0.5 h, 2 h, 12 h and 24 h of growth regulator treatment.

Similarly, the plants for GA-biosynthesis genes expression analyses were prepared. After five days, the plants growing in trays filled with perlite and placed in a growth chamber, were sprayed with a solution of growth regulators. Two days after retardants treatment the tissue was collected, immediately frozen in liquid nitrogen and the genomic RNA was isolated.

Preparation of plant material and RNA isolation. Total RNA was isolated using a commercial RNeasy

Plant Mini Kit (Qiagen), according to manufacturer's instruction. Each sample was analyzed in two biological replications. Obtained genomic RNA preparations were qualitatively and quantitatively evaluated. The evaluation of concentration and purity was performed using the NanoDrop 2000 spectrophotometer. The integrity of the RNA was confirmed by electrophoresis on a 2% agarose gel with 0.01% ethidium bromide.

RT-PCR – preliminary molecular analysis. Reverse transcription to cDNA was carried out using "RevertAid™ H Minus First Strand cDNA Synthesis Kit" (Fermentas). 12 µl of RNA solution and 0.5 µg oligo(dT)$_{18}$ primer or 0.2 µg random hexamer primers were taken. Mixture was incubated for 5 min in 70°C. After chilling, samples were placed in icebath and the following components were added: reaction buffer (25 mM Tris-HCl, pH 8,3; 250 mM KCl; 20 mM $MgCl_2$, 5 mM DTT (dithiothreitol)), 1 µl ribonuclease inhibitor – RiboLock™ (20 µl) and 2 µl 10 mM dNTP mix. Obtained mixture was incubated in 37°C. After this incubation to each sample 1 µl of reverse transcriptase RevertAid™ H Minus M-MuLV (200 U/µl) was added. Final volume of mixture was 20 µl. Obtained mixture was incubated for 60 min in 42°C, after this time reaction was stopped by thermal enzyme inactivation in 70°C for 10 min.

Obtained cDNA was the template for amplification. Directly before PCR 1 µl of polymerase *Taq* (1 U/µl) (Fermentas) was added. PCR reaction was performed in T1 Biometra thermocycler. Cycling parameters were as follows: 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 42°C for 2 min, 72°C for 30 s, and final extension step: 72°C for 5 min. PCR products were visualized on 2% agarose gel stained with ethidium bromide.

Reverse transcription of RNA and real-time analysis. For each form 1 µg of the isolated RNA was reverse transcribed into cDNA using a Superscript VILO cDNA Synthesis Kit (Life Technologies) according to the procedure recommended by the kit manufacturer. The resulting cDNA was the template for the quantitative analysis of gene transcripts by means of the qPCR technique.

Putative reference gene validation. To perform qPCR reactions, it was necessary to determine appropriate reference gene with stable expression in all samples analyzed. Ten putative reference genes were selected for validation (tab. 1). Five of these were reference genes commonly used in the analyses of plant gene expression (actine, α- i β-tubuline, ubiquitin and GAPDH), and five were genes with the most stable expression in wheat seedlings, based on information from the Genevestigator database (www.genevestigator.com). The gene with the most stable expression throughout the experiment was determined by means of the geNorm tool implemented in Biogazelle qbase+ software [Hellemans et al. 2007].

Real-time PCR analysis. As template 80 ng of the cDNA for each reaction was used. All assays were performed in three technical replicates and based on the use of an intercalating SYBR Green dye using SYBR Select Master Mix (Life Technologies) kits. Primers for analyzed genes were designed using Primer3 software [Untergasser et al. 2012]. To confirm the specificity and accuracy of the amplification for each reaction dissociation curve analysis was carried out.

In the next step transcript levels were determined based on the sequence-specific primers and TaqMan probes. Primers and probes were designed using Primer3 software [Untergasser et al. 2012]. TaqMan probes were labeled with FAM as a reporter dye and BHQ1 as a quencher. For qPCR, TaqMan Gene Expression Master Mix (Applied Biosystems) was used. Single reaction was performed in a 20-µl volume, containing 1× TaqMan Gene Expression Master Mix, 250 nM of TaqMan probe and 250 nM of each primer.

Statistical analysis. Statistical analysis of the obtained results during real-time PCR reaction was performed according to the procedure by Yuan et al. [2006]. Analysis of the significance of differences between the values obtained for the ΔC_T form of control, compared to the form of the test. For this purpose, non-parametric Wilcoxon test was used, and the inference was based on the P-value, on significance level $\alpha = 0.05$. For the statistical analysis software v.9.2 SAS was used (SAS Institute).

RESULTS AND DISCUSSION

Preliminary RT-PCR analysis. After reverse transcription, cDNA obtained from analyzed 'Bezostaya 1' *Rht12* line was amplified in PCR reaction with oligo$(dT)_{18}$ primers. After products separation on 2% agarose gel enhancement of cDNA fraction was observed, independent from the time of growth regulator activity. After CCC treatment, the strongest effect was observed on the area between 50 bp and 400 bp. Moreover, 2000 bp DNA fragment was enhanced after 30 min, 2 h and 12 h. Obtained results show that chlormequat chloride influenced on expression of many genes probably connected with gibberellins metabolism pathway. After trinexapac-ethyl treatment enhancement of DNA fragments between 100 bp and 500 bp was observed. Additionally, we observed 1500 bp band after 30 min, 2 h, 12 h, 24 h and 2000 bp fragment after 30 min, 2 h and 12 h.

Table 1. Putative reference genes analyzed in the study

UniGene identifier	Gene product	geNorm M
Ta.54825	actin	0.287
Ta.25534	α-tubulin	0.457
Ta.44405	β-tubulin	0.135
Ta.50503	ubiquitin	0.256
Ta.16204	glyceraldehyde-3-phosphatase dehydrogenase (GAPDH)	0.361
Ta.35546	E3 ubiquitin protein ligase RING-1-like	0.134
Ta.6863	uncharacterized protein	0.235
Ta.14353	protein phosphatase inhibitor	0.220
Ta.9891	nitrogen regulatory protein P-II homolog	0.317
Ta.6591	pleckstrin homology domain-containing family A member	0.160

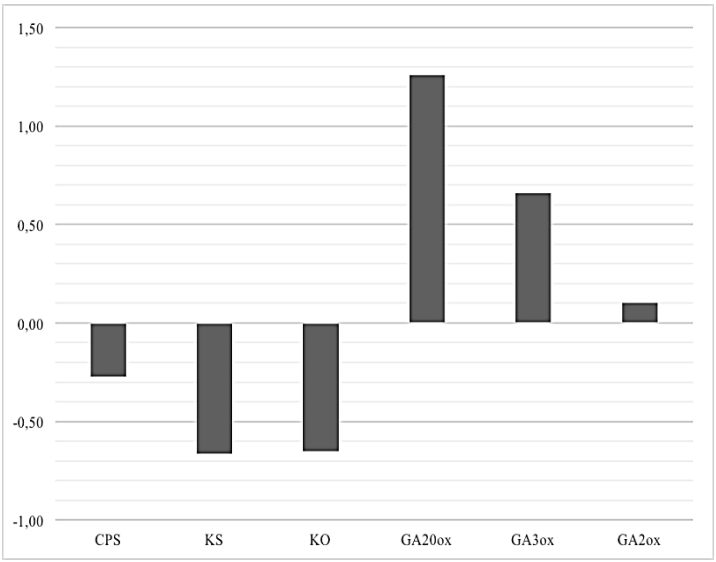

Fig. 1. Fold change of the gibberellin biosynthesis related genes expression in 'Bezostaya 1' *Rht12* line in comparison to tall control form

Reference gene selection. The analysis of the putative reference genes showed very high stability of expression in the analyzed samples, with an average geNorm M value ≤0.2 (tab. 1). The E3 ubiquitin protein ligase RING-1-like gene, which showed the most stable expression, was selected as a reference gene in the subsequent qPCRs.

Expression profile of gibberellin biosynthesis genes in the analyzed wheat Bezostaya *Rht12* line. Analysis of expression of the genes encoding the GA20 and GA3 oxidase (enzymes directly involved in conversion of the precursor forms of the active phytohormones) showed that for wheat line containing the *Rht12* dwarfing gene expression of these genes was higher than in the high control line. For GA2 oxidase gene, responsible for the inactivation of bioactive gibberellin forms, significant increased of expression level was found in analyzed line. The level of expression of this gene was 1.22× higher compared to control form. For other genes encoding enzymes involved in the biosynthesis pathway of gibberellin there were no significant changes in the expression level compared to the control form (fig. 1).

Influence of plant growth regulators on GA biosynthesis pathway genes expression. A significant increase of CPS transcripts level after CCC application was observed in line containing *Rht12* gene in comparison to control line. Moreover slight increase of the CPS gene transcripts level after trinexapac-ethyl application was observed in line with and without *Rht12* gene. Nonetheless, no significant differences in the CPS gene expression level between 'Bezostaya' *Rht12* and Bezostaya *rht* after trinexapac-ethyl application was noticed (fig. 2).

The application of chemical growth regulators induced changes in the expression of the gene encoding ent-kaurene synthase (KS). After application of the CCC an increase of KS gene expression level in control ('Bezostaya' *rht*) line was observed, whereas in dwarf ('Bezostaya' *Rht12*) line transcript level of this gene was decreased. Significant differences in KS gene expression level were observed in control and dwarf line containing *Rht12* gene. In both lines, the expression of this gene was decreased. The line containing *Rht12* dwarfing gene also showed a significant reduction of transcripts level of the gene in the tested tissue after trinexapac-ethyl application compared to the control form (fig. 3).

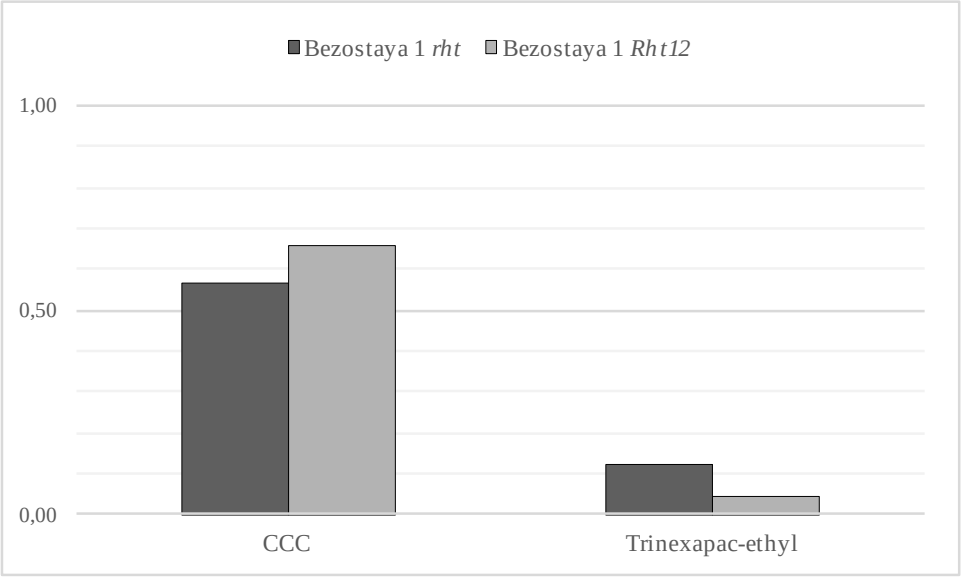

Fig. 2. The influence of plant growth regulators application on CPS gene expression in analyzed common wheat lines

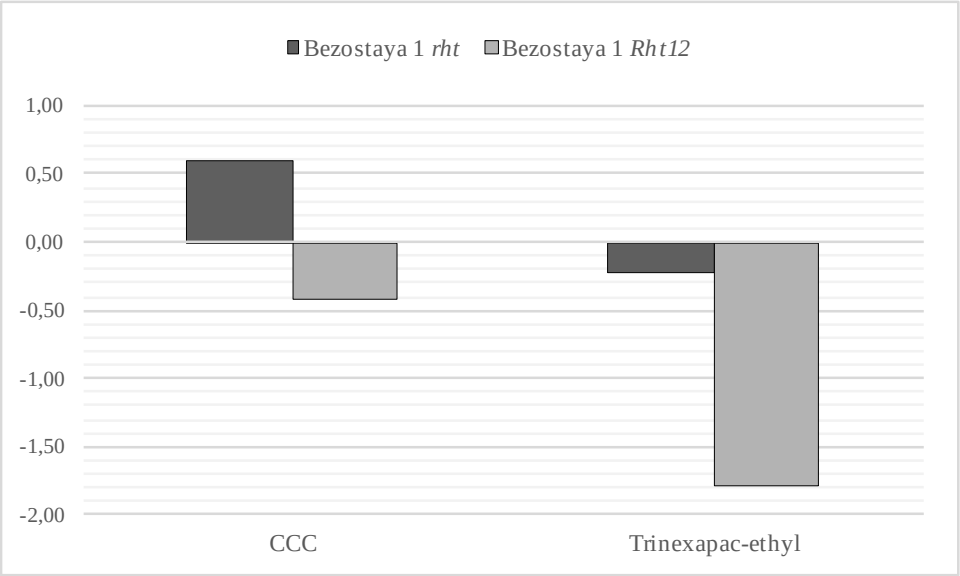

Fig. 3. The influence of plant growth regulators application on KS gene expression in analyzed common wheat lines

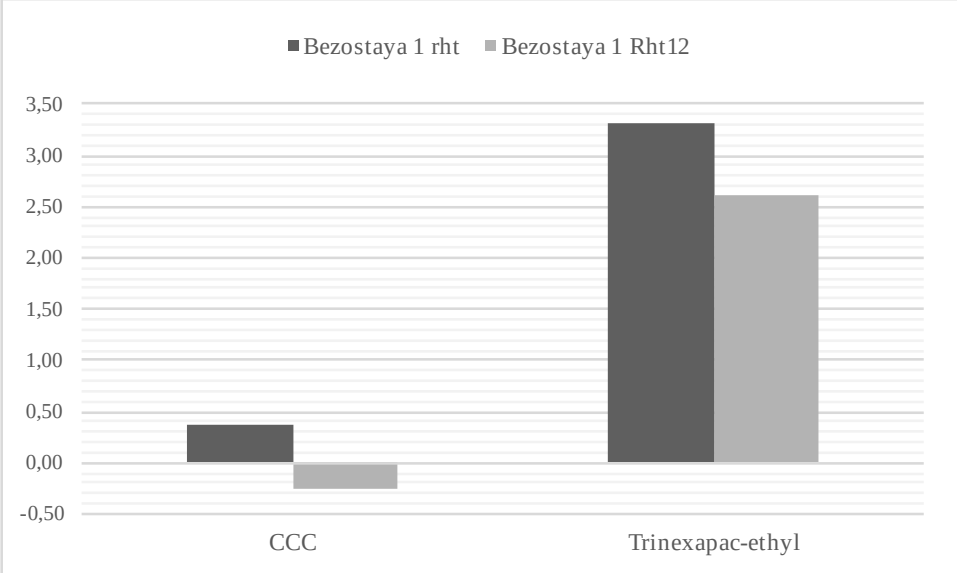

Fig. 4. The influence of plant growth regulators application on GA20ox gene expression in analyzed common wheat lines

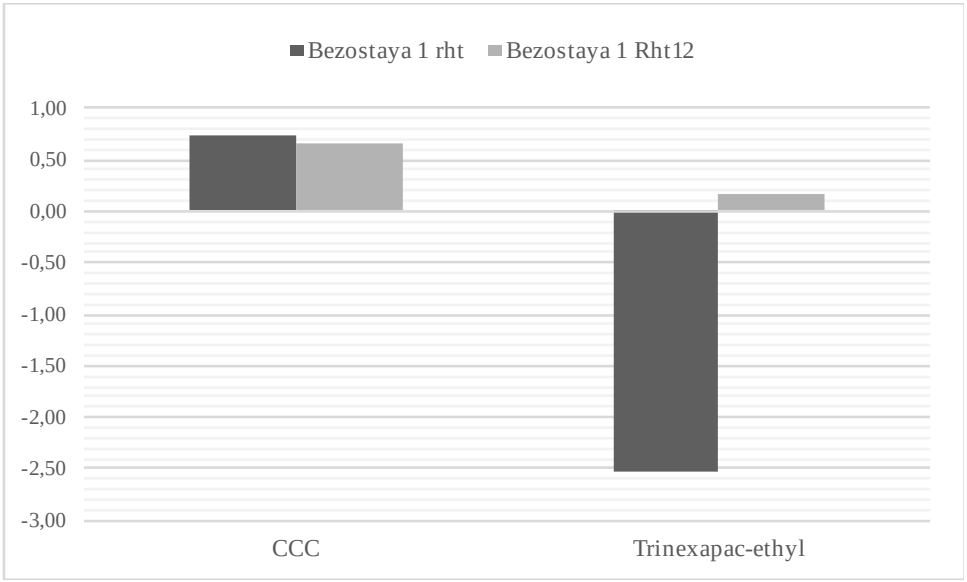

Fig. 5. The influence of plant growth regulators application on GA3ox gene expression in analyzed common wheat lines

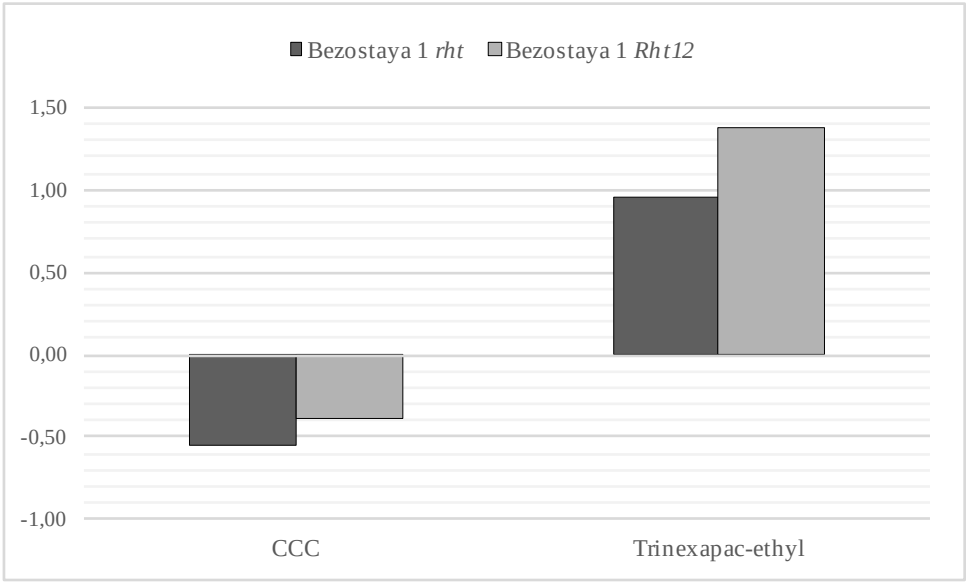

Fig. 6. The influence of plant growth regulators application on GA2ox gene expression in analyzed common wheat lines

Analysis of expression of the gene encoding GA20-oxidase after plants exposure to growth regulators has shown that the action of chlormequat chloride does not cause significant alteration of GA20ox gene expression in line containing the *Rht12*. The expression level of GA20ox gene in dwarfing and control lines after the application of CCC was similar. Different results were obtained after trinexapac-ethyl application. Application of this compound resulted in a strong enhancement of GA20ox gene expression both in control and tested lines. Nonetheless in the 'Bezostaya' *Rht12* line transcripts level of this gene was lower than in the control form (fig. 4).

Analysis of GA3ox gene expression in the tested dwarfing wheat line showed that the application of the CCC solution caused an increase of GA3ox transcripts level lines analyzed. Trinexapac-ethyl application resulted in a strong inhibition of GA3ox gene expression in control form (2.52 ×). For *Rht12* line GA3ox gene expression level did not change significantly compared to untreated form (fig. 5).

Analysis of GA2ox gene expression in response to chemical retardants treatment showed that the response to the CCC action resulted of decrease of transcripts level in the studied lines of wheat. Application of trinexapac-ethyl resulted in upregulation of expression of a gene of interest. The increase in the expression of this gene for the control lines (Bezostaya *rht*) was 0.95×, and for the line containing *Rht12* gene 1.37× (fig. 6).

Plant height is one of the most important traits of wheat, because of its influence on lodging resistance, harvest index and yield [Zanke et al. 2014]. In the wheat semidwarf mutants, plant height reduction is conferred through alterations in growth, mediated by the GA phytohormones. These plant hormones have a well-characterized role in controlling stem elongation, as illustrated by the reduced stature of GA biosynthetic and signaling mutants in many plant species [Pearce et al. 2011]. Inhibition of stem elongation in forms containing GA_3-insensitive alleles is caused by limiting the plant response to phytohormone gibberellin. *Rht-B1* and *Rht-D1* genes encoding modified DELLA proteins which are responsible for inhibiting the growth of plant in response to gibberellin. In contrast, forms containing *Rht-B1b* and *Rht-D1b* alleles produce more effective growth repressors. This effect is caused by substitution which leads to premature STOP codon creation and cut off that part of DELLA protein that is associated with a response to gibberellin.

Rht12 has been classified as a GA-responsive dwarfing gene, but its role, if any, in GA biosynthesis or signaling remains unknown [Chen et al. 2013]. The purpose of our study was to identify and characterize relationship between the presence of *Rht12* gene in wheat genome and the alteration of expression of the genes involved in gibberellin biosynthesis and metabolism pathway. Our research showed that GA20ox and GA2ox transcripts level was higher than in control form. This suggests that the *Rht12* gene is linked to overexpression of a gene associated with both the synthesis and degradation of the active forms of gibberellins. This relationship may be due to the feedback regulating hormone levels in the cells. Analogous results on GA20ox gene expression showed in their studies Hedden and Kmiya [1997].

For analysis of genes associated with plant height semi-quantitative method (RT-PCR, Microarray) were used. Modifying the expression of genes encoding GA using retardants and other factors affect the activity of genes that are regulated by GA. Effects of GA on plant growth and development are mediated through gene expression modulation as RNA and protein synthesis inhibitors interfere with these processes. Microarrays provide high-throughput, simultaneous analysis of mRNA for hundreds and thousands of genes. A transcripts profiles of genes regulated by GA was used by Jan and Komatsu [2006] to understand how GA affect the other genes.

Moons [2008] tested the effects of growth regulators, redox perturbations and weak organic acid stress on PDR family gene expression in rice using RT-PCR. Over half of the PDR genes were JA-induced in roots of rice. Salicylic acid, involved in plant pathogen defense, markedly induced the expression of OsPDR20.

The data, presenting the influence of the application of growth retardants on different plant traits, e.g., yield [Oswalt et al. 2014], stress tolerance [Xu and

Huang 2012], grain quality [Kondhare et al. 2014], lignin accumulation [Peng et al. 2014] or secondary metabolites profile [Giannakoula et al. 2012] have been published in many articles. Notwithstanding, the relationship between the use of PGRs and alterations in GA biosynthesis pathway have not been well described at the molecular level.

The results obtained in the study using RT-PCR confirmed the above results. That is suggested by an enhancement of signal, covering wider area observed on agarose gel. This effect was observed both after CCC and trinexapac-etyl treatment.

Dennis et al. [1965] showed that AMO-1618 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride and other quaternary ammonium-type inhibitors inhibit ent-kaurene synthesis. Moreover, growth inhibition by these chemicals could be reversed by application of GAs, they were thought to function as anti-gibberellins, and they were found to inhibit GA biosynthesis in the fungus [Kende et. al 1963, Hedden and Sponsel 2015].

RT-PCR method was extensively used in the study of cereal plants [Zou et al. 2002, Boutrot et al. 2005, Rampino et al. 2006].

Zou et al. [2002] developed a RT-PCR protocol for studying gene expression during plant embryogenesis. The method could detect genes expressed during embryogenesis at single-embryo level, and therefore can be used to identify phenotypes. Authors used oligo(dT) primers for the reverse transcription step. Then they used primers specific for four genes in *Arabidopsis*: *GAPC*, *STM*, *MP* and *ASK*η. For *GAPC* gene they obtained about 600 bp product, for *MP* about 450 bp, for *STM* about 300 bp and for *ASK*η about 500 bp. Devising method using single-embryo level allows study of the expression dynamics of very weakly expressed genes.

Boutrot et al. [2005] examined *nsLtp* genes expression in developing kernels of *Triticum aestivum* and *Triticum durum*. These genes encode unspecific proteins which transport lipids. After RNA isolation and reverse transcription carried out cDNA amplification using oligo$(dT)_{18}$ primers. After products separation on agarose gel they observed distinct 200 bp and 900 bp bands. Authors obtained that expression of *nsLtp* genes are modified on different levels of kernel development.

Rampino et al. [2006] characterized a mutant of durum wheat (*Triticum durum*) cv. Trinakria by delayed leaf senescence using RT-PCR. They confirmed the altered expression profiles of genes during natural and artificially induced senescence. cDNA was amplified using specific primers for analyzed genes. After separating reaction products on agarose gel they observed differential bands. Based on these products they identified genes connected with leaf senescence.

CONCLUSIONS

The results of the study confirm the association of the dwarfing genes presence in wheat genome with the gibberellin biosynthetic pathway. Analysis of expression of main genes from this pathway in the line containing the *Rht12* gene showed a decrease of CPS, KS and KO transcripts level, and its increase for GA20ox, GA2ox and GA3ox in comparison to the tall line. The reaction was different for the individual genes of the pathway.

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