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The mvp2 mutation affects the generative transition through the modification of 1 transcriptome pattern, salicylic acid and cytokinin metabolism in Triticum 2 3 monococcum 4 Ákos Boldizsár¹, Radomíra Vanková², Aliz Novák^{1,3}, Balázs Kalapos^{1,3}, Zsolt Gulyás^{1,3}, 5 Magda Pál¹, Kristyna Floková⁴, Tibor Janda¹, Gábor Galiba^{1,5,*}, Gábor Kocsy^{1,3} 6 7 ¹ Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, 8 Martonvásár, 2462, Hungary 9 ² Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Academy 10 of Sciences of the Czech Republic, 165 02 Prague 6, Czech Republic 11 12 ³ Doctoral School of Molecular and Nanotechnologies, Research Institute of Chemical and 13 Process Engineering, Faculty of Information Technology, University of Pannonia, Veszprém,

- 14 8200, Hungary
- 15 ⁴ Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and
- Agricultural Research, Institute of Experimental Botany AS CR & Faculty of Science,
 Palacký University, CZ-78371 Olomouc, Czech Republic
- ⁵ Doctoral School of Animal and Agricultural Environmental Sciences, Department of
- 19 Meteorology and Water Management Georgikon Faculty, University of Pannonia, Keszthely,
- 20 8360, Hungary
- 21 ^{*}Corresponding author
- 22

Ákos Boldizsár	boldizsar.akos@agrar.mta.hu
Radomíra Vanková	vankova@ueb.cas.cz
Aliz Novák	novak.aliz@agrar.mta.hu
Balázs Kalapos	kalapos.balazs@agrar.mta.hu
Zsolt Gulyás	gulyas.zsolt@agrar.mta.hu
Magda Pál	pal.magda@agrar.mta.hu
Kristyna Floková	kristyna.flokova@yahoo.fr
Gábor Galiba	galiba.gabor@agrar.mta.hu
Tibor Janda	janda.tibor@agrar.mta.hu
Gábor Kocsy	kocsy.gabor@agrar.mta.hu

1 1 Summary

2 Wild type and *mvp2* (maintained vegetative phase) deletion mutant *T. monococcum* plants 3 incapable of flowering were compared in order to determine the effect of the deleted region of 4 chromosome 5A on transcript profile and hormone metabolism. This region contains the vernalization1 (VRN1) gene, a major regulator of the vegetative/generative transition. 5 6 Transcript profiling in the crowns of T. monococcum during the transition and the subsequent 7 formation of flower primordia showed that 306 genes were affected by the mutation, 198 by 8 the developmental phase and 14 by the interaction of these parameters. In addition, 546 genes 9 were affected by two or three factors. The genes controlled by the deleted region encode 10 transcription factors, antioxidants and enzymes of hormone, carbohydrate and amino acid 11 metabolism. The observed changes in the expression of the gene encoding phenylalanine 12 ammonia lyase (PAL) indicated the effect of mvp2 mutation on the metabolism of salicylic 13 acid, which was also corroborated by the differences in 2-hydroxycinnamic acid and cinnamic 14 acid contents in both of the leaves and crowns, and in the concentrations of salicylic acid and 15 benzoic acid in crowns during the vegetative/generative transition. The amount and ratio of 16 active cytokinins and their derivatives (ribosides, glucosides and phosphates) were affected by 17 developmental changes as well as by mvp2 mutation, too. The absence of VRN1 and other 18 neighbouring genes in the mvp2 mutant plants resulted in the modification of the transcriptome, salicylic acid and cytokinin levels, which changes contributed to the 19 continuous maintenance of the vegetative phase. 20

21 2 Keywords

22 cytokinin, *mvp2* mutation, *Triticum monococcum*, vegetative/generative transition,
23 vernalization

- 24 3 Abbreviations
- 25 Genotypes:
- 26 Tm wt or wt: Triticum monococcum wild type; mvp2: Triticum monococcum maintained
- 27 vegetative phase mutant;
- 28 Salicylic acid metabolism:

29 CA: cinnamate; oHCA: 2-hydroxy-cinnamic acid (ortho-hydroxy-cinnamic acid); BA:

- 30 benzoate; SA: salicylate;
- 31 Light conditions:
- 32 *Ppd*: photoperiod response gene; SD: short day; LD: long day;

1 Development:

VP 20 °C: vegetative phase at 20 °C, single ridge structure of the apices; VP 4 °C: vegetative
phase at 4 °C; DR: double ridge, this phenophase shows the vegetative/generative transition
during the development; SI: initiation of spike primordia;

5 Cytokinins:

6 CK: cytokinin; tZ: *trans*-zeatin; DHZ: dihydrozeatin; iP: isopentenyladenine; cZ: *cis*-zeatin;

7 tZR: *trans*-zeatin riboside; DHZR: dihydrozeatin riboside; iPR: isopentenyladenosine; cZR:

8 cZ riboside; tZR5'MP: tZR 5'- monophosphate; DHZR5'MP: DHZR 5'-monophosphate;

9 iPR5'MP: iPR 5'-monophosphate; cZR5'MP: cZR 5'-monophosphate; tZ9G: tZ-N9-

11 tZOG: tZ-O-glucoside; tZROG: tZR-O-glucoside; DHZOG: DHZ-O-glucoside; DHZROG:

glucoside; DHZ9G: DHZ-N9-glucoside; iP9G: iP-N9-glucoside; cZ9G: cZ-N9-glucoside;

12 DHZR-O-glucoside; cZOG: cZ-O-glucoside; cZROG: cZR-O-glucoside

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14 **4 Introduction**

The exact flowering time is of key importance for perennial grasses since their reproductive organs are highly sensitive to low temperature. Winter wheat necessitates an exposure to low temperature to fulfil its vernalization requirement and ensure its transition from the vegetative to the reproductive phase. Without cold treatment, winter wheat genotypes are incapable of flowering, while spring genotypes do not have such demands. If the transition to the generative phase occurs too early, even a milder frost may result in great yield loss due to the considerable decrease of freezing tolerance.

22 Flowering time is controlled by three well characterized gene families (Laurie, 1997). 23 Photoperiod response genes (*Ppd*) sense day length, and usually, long day (LD) conditions 24 induce their expression. The second gene family contains 'earliness per se' factors, which 25 take part in the initiation of floral primordia and in the determination of the numbers of 26 vegetative and generative primordia independently of environmental conditions (Worland, 27 1996). The third family consists of the vernalization genes VRN1, VRN2 and VRN3, which are 28 inducible by low temperature. Their allelic differences and interactions are important in the 29 timing of the vegetative/generative transition (see for review Distelfeld et al. 2009; Galiba et 30 al. 2009). During vernalization at low temperature, VRN1 is induced, which inhibits the 31 flowering repressor, VRN2. In consequence, the inhibition imposed by VRN2 on VRN3, an 32 activator of flowering, is terminated, and the vegetative/generative transition occurs. The role 33 of VRN1 region in the induction of flowering was demonstrated by using maintained

1 vegetative phase (mvp2) mutant that never flowers (Shitsukawa et al., 2007b). The effect of 2 mvp2 mutation on transcriptome was investigated after one week cold period when the 3 seedlings were still in the double ridge stage (Diallo et al., 2014). Genes related to 4 transcriptional regulation, sugar metabolism, oxidative and biotic stresses were affected by the 5 mutation. However, no transcriptomic data are available during the formation of spikelet primordia. Detail analysis revealed that besides the VRN1 gene, the AGAMOUS-LIKE GENE 6 7 1 [AGLG1; control of fruit development (Yan et al., 2003)], the CYSTEINE PROTEINASE 8 (CYS; degradation of proteins) and PHYTOCHROME-C (PHY-C) genes were also deleted in 9 the *mvp2* mutant. The latter gene encodes a photoreceptor that affects also flowering in a light-dependent way (Chen et al., 2014; Distelfeld and Dubcovsky, 2010). 10

11 Besides the above mentioned major regulators of vernalization, the expression of a large gene 12 set changes during the induction of this process by low temperature as shown by 13 transcriptome analysis in wheat (Gulick et al., 2005; Majláth et al., 2012; Monroy et al., 2007; 14 Winfield et al., 2009). Comparison of the transcriptome profile in a spring and winter wheat 15 genotype during cold treatment showed different expression of genes encoding among others protein kinases, putative transcription factors and Ca-binding proteins (Gulick et al., 2005). 16 17 Winfield et al. (2009) monitored the developmental-phase-dependent gene expression 18 changes and identified several MADS-box genes, which may play an important role in the 19 onset of flowering. The investigation of cold-induced transcript profile changes in 20 chromosome 5A substitution lines ensured the possibility of obtaining more information 21 about the control of flowering since the VRN1 gene is localised on this chromosome. The 22 alterations of the transcriptome of plants in vegetative stage have been compared in winter 23 and spring line, and expression of the gene encoding *Dem* (deficient embryo and meristem) 24 protein affecting the development of apical meristem was proved to be different (Kocsy et al., 25 2010).

26 Plant growth regulators, especially gibberellins (Mutasa-Gottgens and Hedden, 2009), play an 27 important role in the control of flowering. The auxins also regulate flowering through the 28 members of the AINTEGUMENTA-LIKE/PLETHORA transcription factor family (Krizek, 29 2011). The role of methyl jasmonate in this process was recently shown in T. monococcum, 30 where its level was higher, and many jasmonate-responsive genes were affected in non-31 flowering *mvp2* mutant (Diallo et al., 2014). In addition, treatment with this hormone delayed 32 flowering with simultaneous downregulation of VRN1 and VRN3 genes. Moreover, salicylic 33 acid (SA) participates also in the control of flowering time since SA-deficient plants flower 34 later and UV-C stress activates the vegetative/generative transition in Arabidopsis through this hormone (Martínez et al., 2004). The involvement of cytokinins as a long-distance signal of the floral transition process has also been recently shown (Bernier, 2013). Transient cytokinin maximum was observed at the onset of vegetative-generative transition both in *Brassica napus* (Tarkowská et al., 2012) and *Triticum monococcum* (Vanková et al., 2014).

5 Despite the intensive study of the regulation of flowering processes, the transcriptional and 6 hormonal control during the initial development of flower primordia in wheat plant is still 7 poorly understood. In the present experiments the possible involvement of the *mvp2* mutation-8 dependent changes of transcriptome profile and the SA and cytokinin metabolism in the 9 control of vegetative/generative transition have been studied.

10 **5** Materials and methods

11 5.1 Plant material and treatments

Triticum monococcum KU 104-1 strain and its *mvp2* mutant were analysed in this study. The *mvp2* mutant was generated by ion beam radiation and has a large deletion that includes *VRN1*(Shitsukawa et al., 2007b) and the other three genes (Distelfeld and Dubcovsky, 2010).

15 After germination in Petri dishes between wet filter papers (1 d 25 °C, 3 d 5 °C, 2 d 25 °C), seedlings were grown with a photoperiod of 16 h (light cycle started at 2:00 and finished at 16 18:00), at 260 µmol m⁻² s⁻¹, 20/17 °C and 75/65% RH in a growth chamber (Conviron PGV-17 15; Controlled Env., Ltd., Winnipeg, Canada). Seedlings were raised in a 2:1:1 (V/V/V) 18 19 mixture of garden soil, humus and sand in wooden boxes (150 plants in a box). Dimensions of 20 the soil blocks in the boxes were $26 \times 38 \times 10$ cm (length \times width \times depth), distance between 21 the plants was 2.5 cm. After 3 weeks, temperature was set immediately to continuous 4 °C 22 (day/night), other environmental parameters remained unchanged. Crown and leaf (the second 23 youngest leaves) samples were taken for hormone measurements (in case of cytokinin 24 analysis, only the leaves were examined) and gene expression studies before the cold 25 treatment; after 2 weeks at 4 °C, when the seedlings were still in the vegetative developmental 26 stage; during the vegetative/generative transition (double ridge stage) and after the appearance 27 of the spikelet primordia (spikelet initiation phase). In the last two sampling points samples 28 for microarray analysis were collected separately from the crowns. Although there was no 29 developmental phase change in *mvp2*, the samples from this genotype were collected at the 30 same time as in the wild type and the same nomenclature was used for the indication of the 31 sampling points (Fig. 1). Each sampling was started after a 6-hour illumination and lasted for 32 60-90 min. The experiments were repeated 3 times. In each experiment, 3 samples consisting

33 of a mixture of the crowns and leaves, respectively, from 9 plants were analysed.

1 5.2 Selection of the *mvp2* mutants by genotyping

2 Leaf samples (100 mg FW) were collected from the seedlings and disrupted by TissueLyser 3 (Qiagen) (shaking settings: 25 Hz, 90 sec). The total DNA was isolated from crushed material 4 by the Zeno-Gene40 DNA isolation kit (Zenon Bio Ltd.) and eluted in 200 µl of elution buffer 5 in the last step. Genotyping was carried out in two-steps. In the first screening step a 1084 bp long segment of the VRN1 gene was amplified by PCR (Applied Biosystems® GeneAmp® 6 7 PCR System 9700) according to the manufacturer's instructions. The reaction mixture 8 contained 2.5 µl 10x Key buffer, 2.5 µl DNA, 0.5-0.5 µl VRN1-specific primers (10 µM, 9 Suppl. table 1), 0.5 µl dNTP mix (2.5 mM), 0.4 µl Red Taq enzyme (Red Taq DNA Polymerase enzyme (VWR)) and 18.1 µl water. The chosen primers amplified the fragment of 10 11 the VRN1 gene not present in the homozygous mutant. The applied PCR conditions were as 12 follows: 40 cycles of 94 °C for 20 sec, 61 °C for 30 sec, and 72 °C for 80 sec. In case of 13 ambiguous samples, the second screening step was employed. According to Dhillon et al. 14 (2010) a modified method was applied. The following primers were used in 10 µM 15 concentration: 1 µl F18, 0.5 µl R23 and 0.75 µl R22. In this multiplex PCR reaction the wild type Triticum monococcum gives two amplicons (172 and 339 bp) while mvp2 mutant gives 16 17 only one amplicon. The sequence of the primers is given in the Suppl. table S1.

18 5.3 Selection of the sampling time according to the morphology of shoot apices

The developmental stage of the shoot apices, isolated from the crowns of the seedlings, was determined under a Zeiss Stemi 2000-C stereomicroscope (Carl Zeiss Mikroskopie, Jena, Germany) according to the scale of Gardner (Gardner et al., 2007) (Fig. 1). Three developmental phases were distinguished according to the wild type: vegetative (VP, single ridge structure, Gardner's stages 0-1), double ridge (DR, vegetative/generative transition, Gardner's stage 3) and generative phases (SI, initiation of spike primordia, Gardner's stages 4-5).

26 5.4 Determination of hormones

27 Methanol-soluble free and bound SA and their precursors were measured according to Pál et 28 al. (2005), who modified the method of Meuwly and Métraux (1993). Benzoic acid (BA) and 29 cinnamic acid (CA) were measured by UV spectrophotometry in the range of 230–300 nm 30 (W996 photodiode array detector, Waters, Milford, MA). SA and *ortho*-hydroxy-cinnamic 31 acid (*o*HCA) were quantified fluorimetrically (W474 scanning fluorescence detector), with 32 excitation at 317 nm and emission at 436 nm for *o*HCA, followed by excitation at 305 nm and 33 emission at 407 nm for SA.

1 Cytokinins were extracted and purified as described earlier (Svačinova et al., 2012), with modifications. Briefly, 1ml 2*10⁻⁸ M concentration of ice cold extraction mixture of 2 methanol/water/formic acid (15/4/1, V/V/V) containing stable-isotope-labelled internal 3 standards of cytokinins (²H₅-tZ, ²H₅-tZR, ²H₅-tZRMP, ²H₅-tZ7G, ²H₅-tZ9G, ²H₅-tZOG, ²H₅ 4 tZROG, ²H₃-DZ, ²H₃-DZR, ²H₃-DZRMP, ²H₃-DZ9G, ²H₇-DZOG, ²H₆-iP, ²H₆-iPR, ²H₆-i 5 6 iPRMP, ²H₆-iP7G, ²H₆-iP9G) was added to homogenized samples and stirred for 30 min/4°C. After centrifugation the supernatants were passed through the Sep-Pak Plus C₁₈ cartridge 7 8 (Waters, Milford, MA, USA) to remove the lipids and pigments. Cytokinins from flow-9 through fraction were concentrated by mixed mode Oasis MCX reverse phase-cation exchange SPE column (Waters, Milford, MA, USA) and eluted with 0.35 M NH₄OH in 60% 10 methanol. Eluates were evaporated to dryness in a Speed-Vac concentrator RC1010 (Jouan, 11 12 Winchester, UK) and reconstituted by 30 µl of mobile phase prior to UHPLC-MS/MS analysis. An Acquity UPLC® System (Waters, Milford, MA, USA) coupled to a triple 13 quadrupole mass spectrometer XevoTM TQ MS (Waters MS Technologies, Manchester, UK) 14 15 with an electrospray interface (ESI) was employed in cytokinin analysis. Compounds were separated on reverse-phase column (Acquity UPLC® BEH C18, 1.7 µm, 2.1 × 150 mm, 16 17 Waters) by 24 min binary gradient consisting of methanol (A) and 15 mM ammonium formate 18 pH 4.0 (B) (Svačinova et al., 2012). The effluent was introduced in the ESI source of the 19 tandem mass spectrometer Xevo TQ MS. Cytokinins were determined and quantified by multiple ion monitoring mode (MRM). MassLynxTM software (version 4.1, Waters, Milford, 20 21 MA, USA) was used to operate the instrument, acquire and process the MS data.

22 5.5 Gene expression studies

Total RNA was isolated using Direct-zolTM RNA Miniprep Kit (Zymo Research) as described 23 24 by the manufacturer. Reverse transcription was carried out with M-MLV reverse transcriptase 25 and Oligo(dT)₁₈ primer (Thermo Scientific) according to the method of the supplier. 1500 ng RNA was transcribed into cDNA in 100 µl final volume. From this cDNA solution, 1 or 1.5 26 µl were utilized for the qRT-PCR analysis. Transcript levels were determined by real-time 27 RT-PCR using a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad). Most primer 28 29 sequences were designed by our group while those of VRN1 were found in the literature 30 (Loukoianov et al., 2005), (Suppl. table S1). In case of all primers, the efficiency ranged between 95 and 100%. Relative transcript levels were calculated by $\triangle \triangle C_t$ method using the 31 32 housekeeping gene similar to phosphoglucanate dehydrogenase protein (unigene identifier: 33 Ta30797) for normalization (Paolacci et al., 2009). In all cases the expression levels were 34 compared to the DR phase of Triticum monococcum wild type (Tm wt). The Bio-Rad CFX Manager 3.1 (File version: 3.1.1517.0823) calculated the relative normalized expression. In the charts, the error bars show the standard deviation and the y-axis was scaled in \log_2 . The outliers were excluded from the analysis in case the standard deviation of C_t value exceeded

4 0.3 in the replicate group.

5 5.6 Microarray experiments

6 For microarray analysis, total RNA was isolated using Qiagen kit (RNeasy Plant Mini Kit).

7 Three parallel samples were isolated from the crowns of Tm wt and mutant plants. 4000 ng

8 total RNA samples were eluted in 40 μ l final volume (100 ng/ μ l). 300 ng were analysed on

9 gel and 200 ng were analysed with Agilent Bioanalyzer Instrument. RNA purity was checked

10 by the absorbance ratios of 260/280 and 260/230 nanometres. Ratio of sample absorbance was

11 2 or higher. Gel electrophoresis confirmed the intactness of the RNAs. Samples with an RNA

12 integrity number (RIN) above 8.0 were used for further analysis.

13 Total RNA (200 ng) was labelled and amplified using Low Input Quick Amp Labelling Kit 14 according to the instructions of the manufacturer (Agilent Technologies). Labelled RNA was 15 purified and hybridized to Agilent Wheat genome 4x44K array slides according to the 16 manufacturer's protocol. After washing, array scanning and feature extraction were performed 17 with default scenario by Agilent DNA Microarray Scanner. Fluorescence intensities of spots 18 were quantified, background subtracted, and dye-normalized by Feature Extraction software, 19 version 9.5 (Agilent Technologies). Data were then imported and analysed using GeneSpring 20 GX program (Agilent Technologies) to generate lists of genes with a minimum 2-fold 21 difference in the expression. Array data are available in ArrayExpress data bank with the ID 22 E-MTAB-3968.

23 5.7 Statistical analysis

For the statistical analysis one-way ANOVA and Dunett t or Tukey B *post hoc* tests were used by SPSS 16.0. Normality was tested by Kolmogorov-Smirnov probe, homogeneity of the variances was tested by Levene's test.

27 6 Theory

The deletion in *mvp2* mutants including the *VRN1* gene is assumed to control the transcript profile changes during the vegetative/generative transition and initial development of the flower primordia. The alterations in gene expression may affect, among others, the metabolism of SA and cytokinins, i.e. hormones influencing flowering.

1 7 Results

2 7.1 Effect of the gene deletion and developmental stage on transcript profile

Based on selection criteria of a minimum 2-fold difference in the expression related to the genotype, developmental stage or their interactions, 1064 were selected from the 43604 genes present on the array (Suppl. Table 2). Comparison of transcript profiles in crowns of wild type and *mvp2* mutant plants during the vegetative/generative transition and the formation of spikelet primordia (Fig. 1) indicated that 198 genes were affected by the developmental phase, 306 by the *mvp2* mutation and 14 by their interactions (Fig. 2). In addition, 546 genes were affected by two or three factors.

10 7.2 Validation of microarray results and clustering of the selected genes

11 Microarray data allowed selecting several cold acclimation- or vegetative/generative 12 transition-related genes that were differentially expressed in the crowns of the two genotypes 13 or during the development. Microarray results were validated by qRT-PCR (Fig. 3). There was a close correlation (R^2 =0.98) between the microarray and qRT-PCR results (Suppl. Fig. 14 S1). The expression of CBF4, CBF14 and R2R3-MYB genes exhibited a strong induction in 15 16 wt plants between the DR and SI phases while it was constitutively high in the mvp2 mutant 17 (Figs. 3 A-C). The transcript level of VRN1 decreased after the vegetative/generative 18 transition in wt while it was not detectable in mvp2 because of the deletion on the 5A 19 chromosome (Fig. 3 D). The expression of the genes encoding a Ser-Thr protein kinase 20 (STPK), LG (lipoxygenase), C2H2 Zn-finger and heat shock proteins (HSP17, HSP80, 21 HSP101) and MPBF1 (multiprotein bridging factor 1) decreased in wt after entering the 22 generative phase while it exhibited a continuously low level during both DR and SI phases in 23 the mutant (Fig. 3 E-K). In case of PAL (phenylalanine ammonia-lyase), a lower decrease was 24 observed in the mvp2 plants in SI compared to the DR phase (Fig. 3 L). Changes in the 25 expression of heat shock proteins (HSP80, HSP17 and HSP101) showed similar tendencies. 26 In the wild type, strong decrease was observed after the vegetative/generative transition and in 27 *mvp2*, low transcript levels occurred at the DR phase which further decreased at SI phase (Fig. 28 3 H-J).

The pool of 1064 genes (in Fig. 2) was further filtered by ignoring those that were functionally not annotated and whose E-values were smaller than 1e-40. Two hundred genes satisfied these criteria (Suppl. Table 2). Relative expression values of wt SI, *mvp2* DR and *mvp2* SI normalised to wt DR were compared by hierarchical clustering. This revealed the genotype-dependency of genes related to stress tolerance and showed that genes related to

1 transcriptional regulation and carbohydrate metabolism were influenced by the developmental 2 phase (Suppl. Fig. S2). After an additional selection, a filtered heat-map was constructed (Fig. 3 4) in which only those 137 genes were kept that exhibited more than 2-fold differences between the genotypes at DR phase (Fig. 4). Approximately one fourth of these genes showed 4 5 higher expression in *mvp2* at the DR stage compared to the same developmental phase of wt, 6 which may indicate their repression by one of the deleted genes (VRN1, AGLG1, CYS and 7 *PHY-C*). Among these genes, several *CBF* and MADS-box transcription factors can be found. 8 In contrast, many HSPs had greater expression in wt, which indicates their induction by the 9 deleted gene(s).

7.3 Assignment of genotype- and development-responsive genes to biological processes,
 molecular functions, KEGG pathways and their networks

12 Different methods were chosen for evaluating the results of the microarray analysis (Suppl. 13 Table 2). First of all, *Arabidopsis* homologs were selected whose E-value \leq 1e-40 and whose 14 expression differences between the compared groups (D, G, and common set of $D \times G$) were 15 higher than or equal to 2 (in log₂ value). After that the genes were analysed by BiNGO 16 Cytoscape plug-in software (Maere et al., 2005) to determine over-represented biological 17 processes or molecular function categories. According to the categorization of biological 18 processes, the first five groups containing the highest number of the genes influenced by the 19 genotype are the metabolic process (GO:8152), primary metabolic process (GO:44238), 20 response to stimulus (GO:50896), response to stress (GO:6950) and biosynthetic process 21 (GO:9058), which contain 22, 19, 16, 15 and 12 genes, respectively (Suppl. Fig. 3). Two of 22 these groups [response to stimulus (GO:50896), response to stress (GO:6950)] contain high 23 number of genes influenced by the developmental phase, too. The following groups 24 containing 7 and 6 genes influenced by D are the response to chemical stimulus (GO:42221) 25 and response to abiotic stimulus (GO:9628), respectively. This indicates that, in our 26 experiment, cold treatment was at least as effective on the gene expression changes as 27 mutation. PAL is one of these genes, which prompted us to determine the SA content and 28 investigate the SA biosynthesis in more detail.

Three molecular function GO categories contain most of the genes from the array: namely, catalytic activity (GO: 3824), oxidoreductase activity (GO:16491) and binding (GO:5488). The first category consists of 34 genes influenced by genotype and 18 influenced by developmental phase, while the second one contains 14 and 6 genes, respectively (Suppl. Fig. 4). Among the genes of the GO: 3824 category influenced by the genotype, the following two have the best E-values: glutamine-dependent asparagine synthetase (AAU89392.1) and

1 spermidine synthase (AHJ14572.1). Both genes are important in stress tolerance. Asparagine 2 synthetase is essential in the synthesis of L-glutamate, the precursor of glutathione and 3 spermidine. Spermidine synthase catalyses formation of spermidine enhancing tolerance against various stresses (Kasukabe et al., 2004). Interestingly, both genes had lower 4 5 expression level in the *mvp2* genotype compared to wild type (Fig. 4 and Suppl. Fig 2). Among the identified oxidoreductase enzymes (GO:16491) ABA 8'-hydroxylase, the key 6 7 enzyme in the catabolism of the abscisic acid (Krochko et al., 1998), had slightly lower 8 activity (-0.25-fold difference) in the mutant genotype (Suppl. Fig. 2 and 4).

9 According to the KEGG pathway cluster analysis the main pathways containing the majority of genes influenced by genotype are general metabolic and secondary metabolites 10 11 biosynthesis pathways followed by the steroid biosynthesis (ATH00100), phenylpropanoid 12 biosynthesis (ATH00940) and phenylalanine metabolism (ATH00360) (Suppl. Fig. 5). The 13 occurrence of the phenylalanine metabolism among the overrepresented KEGG pathways 14 confirmed importance of the thorough examination of SA biosynthesis in order to determine 15 the differences between the genotypes. However, the thorough analysis of the other processes 16 (such as steroid biosynthesis and phenylpropanoid biosynthesis) exceeds the scope of this 17 study.

18 Fig 5. shows the network of proteins encoded by the selected 59 genes (Suppl. Table 2 row C). 19 Additional proteins interconnecting the former ones with proteins encoded by the four deleted 20 genes were included. Protein-protein interactions were identified using the BioGRID 21 (Oughtred et al., 2016) database, and were visualised by Osprey software (Breitkreutz et al., 22 2003). Gene selection was based on the homology between the Triticum aestivum and 23 Arabidopsis. The deleted genes in mvp2 were very close to each other (Yan et al., 2003). In wt 24 VRN1 and AGLG1, genes are next to each other and the other two genes are also localized 25 close to Vrn1 locus. In our map, instead of AGLG, SEP4 and, instead of CYS, SAG12 genes 26 are presented because of the better E-values of the annotated sequences. In this network, three 27 of the four deleted genes form a close linkage. From among the closest genes, 6 were present 28 on the array and three of them, AGL14, AGL20 (similar to SOC1) and AGL19, were induced 29 because of the lack of VRN1 (synonym name: AP1), SEP4 and SAG12. SOC1 (suppressor of 30 overexpression of CO1) gene causes delayed flowering, according to the examination of 31 mutant Arabidopsis (Onouchi et al., 2000), and it was determined in wheat that WSOC1 acts 32 upstream of WAP1 (wheat AP1 = VRN1) or regulates the flowering in a different pathway 33 from the WAP1 (Shitsukawa et al., 2007b). In Arabidopsis there are three flowering 34 regulatory pathways (long-day-, vernalization- and autonomous flowering pathways). SOC1 is

connected to the first one, but these pathways are partially redundant. The expression of this
 gene is highly induced in the mutants that never flowers (Fig. 5). Our results are in
 accordance with the reports of Shitsukawa et al. (2007a, 2007b).

4 7.4 Effect of *mvp2* mutation and developmental stage on SA and cytokinin levels

5 SA can be synthesized in three different ways; besides SA itself, three of its possible 6 precursors, cinnamic acid (CA), 2-hydroxy-cinnamic acid (oHCA) and benzoic acid (BA), 7 were measured in the crowns and leaves of the wt and mutant plants before and after a two-8 week treatment at 4 °C, at the DR and SI stages (Fig. 6). The bound forms of all compounds 9 were present in a much higher concentration than the free forms both in the crowns and 10 leaves. In *mvp2* crowns at the VP at 20 °C, free *o*HCA, free BA and free SA were not present 11 in detectable concentrations (Fig. 6 A, G and E). Exposure to low temperature increased the 12 oHCA levels in the leaves and crowns, BA in the leaves in both genotypes, the levels of SA in 13 the leaves and CA in the crowns in the mutant plants. At DR phase the bound forms of all 14 measured compounds in the crowns were present in higher concentrations in the mvp2 mutant 15 than in wt plants (Fig. 6 A, C, E, G). Similar differences were observed in the leaves for 16 oHCA and CA (Fig. 6 B and D). The differences between the genotypes were eliminated after 17 the transition to the generative phase at SI. There were no alterations either in the crowns or in 18 the leaves except in case of SA and CA in crowns where the amounts of free forms were 19 greater in the mutant plants (Fig. 6 C and E).

20 Contents of cytokinin metabolites were compared in wt and *mvp2* leaves during the prolonged 21 cold exposure associated in wt with developmental transition. General response of both 22 genotypes to low temperature was down-regulation of the levels of precursors of active 23 cytokinins, i.e. cytokinin phosphates (Suppl. Fig. 7C), which indicated suppression of 24 cytokinin biosynthesis under non-optimal temperature. Physiologically the most active 25 cytokinin is trans-zeatin. The biosynthesis of this cytokinin, presented according to the 26 KEGG database, is shown in Suppl. Fig. 6 where all determined cytokinin metabolites are 27 highlighted. Its isomer, *cis*-zeatin, which prevails in some monocots, is less active in the 28 stimulation of cell division (by more than one order of magnitude), but maintains other CK 29 functions and seems to be associated with stress responses. Apart of trans-zeatin and cis-30 zeatin, active cytokinins include also isopentenyladenine and dihydrozeatin (Suppl. Fig. 7A). 31 The corresponding ribosides were reported to be predominantly transport forms (Lomin et al., 32 2015). Two-week long cold treatment had positive effect on *trans*-zeatin and its riboside in 33 wt. In case of the mutant, low temperature resulted in decrease of isopentenyladenine, 34 dihydrozeatin and *cis*-zeatin, while *trans*-zeatin riboside and isopentenyladenosine increased

1 (Fig. 7). The maximum of active cytokinins were found at the DR stage, i.e. during 2 vegetative/generative transition. In wt, the predominant cytokinin was *trans*-zeatin, in mutant, 3 trans-zeatin riboside and isopentenyladenosine prevailed. SI stage was associated with substantial drop of active cytokinins, especially in wt, which undertook successful 4 5 developmental transition. Mutant preserved higher levels of isopentenyladenosine. The 6 mutant constitutively remaining in vegetative stage exhibited decreased levels of cytokinin deactivation products - cytokinin N- and O-glucosides, especially of trans-zeatin-N9-7 8 glucoside, dihydrozeatin-N9-glucoside, cis-zeatin-N9-glucoside, trans-zeatin-O-glucoside, 9 cis-zeatin-O-glucoside (Fig. 7). This may indicate diminished cytokinin biosynthesis or much 10 slower turn-over of active cytokinins. In wt, concentration of cytokinin N- and O-glucosides 11 exhibited a transient increase during DR, and then it decreased (Suppl. Figs. 7 D, E).

Thus, as shown in Suppl. Fig. 7, highly significant changes of cytokinins were observed during the vegetative/generative transition. Developmental phase affected content of *trans*zeatin associated with stimulation of cell division. Precise regulation of active cytokinin levels is reflected by elevation of *trans*-zeatin-riboside, *trans*-zeatin O-glucoside, *trans*-zeatin Nglucoside, *cis*-zeatin O-glucoside.

17 8 Discussion

18 8.1 Effect of *mvp2* mutation and generative transition on the gene expression

19 The transcriptome analysis confirmed our hypothesis that *mvp2* mutation affects the 20 vegetative/generative transition through the modification of the transcriptome profile since the 21 expression of 306 gene differed in the mutant compared to the wild type plants. Several genes 22 encoding agamous-like (AGL) MADS box transcription factors are possibly under the 23 negative control of the protein encoded by one of the deleted genes as it was shown by their 24 greater expression in the mutant. Based on our network of the proposed protein interactions 25 (Fig. 5), the negative regulator is probably the VRN1 (AP1) protein that connects to several 26 AGL proteins and is a major regulator of flowering. These AGL transcription factors control 27 flowering at several levels including the timing of the generative transition and determination 28 of floral meristem identity (Lee et al., 2000; Winfield et al., 2009; Yu et al., 2002). The 29 freezing tolerance-related CBF genes were also inhibited by the deleted region since their 30 expression was greater in the deletion mutant. This finding corroborates previous observations 31 showing the coordinated control of the generative transition and freezing tolerance (Dhillon et 32 al., 2010; Diallo et al., 2014; Galiba et al., 2009). The proteins encoded by the region deleted 33 in the *mvp2* mutant proved to be positive regulators of several transcription factors (WRKY,

1 C2H2 Zn-finger) and heat shock proteins (HSP17, HSP80, HSP101) as indicated by the low 2 expression of the corresponding genes in the mutants. The observed effect on HSPs gives a 3 further evidence for the coordinated regulation of stress response and flowering. Gene 4 ontology analysis based on biological processes indicated the effect of *mvp2* mutation on 5 amino acid metabolism-related genes, while analysis of molecular functions showed the 6 control of oxidoreductases and monooxygenases by the deleted chromosome region. The 7 effect of whole chromosome 5A on amino acid metabolism at transcript and metabolite levels 8 was shown in previous studies (Juhász et al., 2015; Kocsy et al., 2010; Kovács et al., 2011) 9 and the present investigations demonstrated that the region with the VRN1 gene is responsible 10 for this effect. The results also indicate the involvement of redox processes in the control of 11 the vegetative/generative transition (Diallo et al., 2014; Gulyás et al., 2014). A comparison of 12 the genotypes related to the KEGG metabolic pathways indicated the effect of the mutation on 13 flavonoid and steroid biosynthesis. These results are in good agreement with previous ones 14 describing the role of flavonoids in the reproductive processes (Falcone Ferreyra et al., 2012). 15 The various analyses of the transcriptome data also revealed the influence of *mvp2* mutation 16 on the genes involved in the SA and cytokinin metabolism that is discussed in the next 17 sections.

18 A large set of genes (198) was affected by the vegetative/generative transition independently 19 of the *mvp2* mutation. These genes are not regulated by those ones located in the deleted 20 region in *mvp2* mutant. Among others, the genes encoding a MYB transcription factor, a 21 peroxidase and a protein kinase were upregulated and those encoding an RNA polymerase, a 22 pre mRNA processing factor and an S-like RNase were down-regulated. This observation 23 indicates the induction of regulatory genes and inhibition of the genes involved in the RNA 24 metabolism during the vegetative/generative transition. Based on gene ontology analyses and 25 alignment to KEGG metabolic pathways, the expression of genes related to carbohydrate 26 metabolism, response to heavy metals, transition metal binding and carbon fixation was 27 affected by the vegetative/generative transition. These results show that the energy 28 homeostasis is modified during the generative transition.

29 8.2 Involvement of SA and cytokinins in the control of generative transition

The differences between wt and mvp2 plants in the amount of SA and its precursors indicate the effect of the *VRN1* region on SA metabolism during the vegetative/generative transition in *T. monococcum*. Low temperature treatment induced certain components of SA biosynthetic routes including *o*HCA, BA and SA in the crowns and/or in the leaves. Earlier findings also showed that low temperature hardening of bread wheat (*T. aestivum*) in the light, but not in

1 the dark, led to a substantial increase in the bound oHCA level (Janda et al., 2007). Since 2 these compounds may also serve as antioxidant compounds (Foley et al., 1999), their role in 3 the maintaining of the redox balance was assumed. In the present work this increase was 4 significant in both wt and *mvp2* plants and was detectable in the bound forms of these 5 components indicating that the levels of free SA and, most probably, of the other related 6 compounds are strictly controlled (Janda et al., 2014). These results indicated that SA 7 significantly affects the initiation of flowering of wheat plants. Substantially lower total 8 oHCA and SA levels were detected in the crowns of non-flowering mvp2 mutant than in the 9 wild-type plants at DR stage. This indicates that although SA is probably necessary for 10 flowering initiation in wheat plants, its later existence is not required anymore. Similar 11 findings were recently published in Arabidopsis plants, where transfer of plants from short- to 12 long-day growth conditions was paralleled with a temporary increase in the SA level at pre-13 reproductive stages followed by a decline at the reproductive stage (Abreu and Munné-Bosch, 14 2009). In spite of the finding, that Arabidopsis thaliana plants impaired in SA accumulation 15 or transport mechanisms flowered later than the wild-type (Martínez et al., 2004), which may 16 indicate direct link between SA and floral development; exogenous treatment of NahG 17 Arabidopsis plants with SA was not able to restore the wt flowering time (Martínez et al., 18 2004). However, it must be also mentioned that exogenous SA may be easily converted to 19 bound form; therefore, the exact level of active SA cannot always be estimated (Szalai et al., 20 2011). The increase of biomass production during the reproductive stage and the final seed 21 production in NahG transgenic and sid2 mutant Arabidopsis plants (both with impaired SA 22 accumulation capacity) in comparison to wild-type plants (Abreu and Munné-Bosch, 2009) 23 may suggest SA function in cold acclimation and elevation of plant stress tolerance 24 (associated with increased levels of protective compounds), which positively affects further 25 development.

26 In cereals, the involvement of cytokinins has been studied during cold hardening (Kosová et 27 al., 2012). Both cold tolerance and tillering ability were greatly increased in transgenic turf 28 grass plants overproducing the cytokinin biosynthetic gene for isopentenyl transferase (Hu et 29 al., 2005). However, less is known about their role in the development of generative phase in 30 crops. The developmental changes are usually paralleled with altered hormonal levels. The 31 elevation of cytokinins (and auxins) is associated with stimulation of meristematic activities 32 in plants. Cytokinins control both check-points during cell cycle progression (Schaller et al., 33 2014). Isopentenyladenine of leaf origin was reported to trigger the floral transition in the 34 shoot apical meristem of Arabidopsis (Bernier, 2013). Increased cytokinin concentration was

1 detected at the onset of vegetative-generative transition in Brassica napus (Tarkowská et al., 2 2012). Comparison of cytokinin profiles in T. monococcum spring line DV92 and winter line 3 G3116 showed that the maximum of active cytokinins occurs at the onset of the early stage of 4 reproductive development (Vanková et al., 2014). Spring line exhibited cytokinin maximum 5 after 21 days, while winter line after 42 days when it fulfilled its vernalization requirement as 6 indicated by the decrease of the frost tolerance and dehydrin levels. These data are in 7 accordance with our results, which showed maximum of active cytokinins in DR stage. In wt, 8 developmental transition was accompanied by subsequent fast down-regulation of cytokinins. 9 During SI stage, when cell elongation and differentiation take place, other hormones, 10 probably auxins and gibberellins, may exhibit their maxima.

11 Cytokinin elevation was also observed in *mvp2* mutant. Accumulation of predominantly 12 trans-zeatin riboside and isopentenyladenosine may indicate tendency to overcome the 13 developmental block in the mutant. Up-regulation of these compounds may indicate cytokinin 14 transport from the roots. It might be also related to the cold acclimation of this genotype. 15 Gradual cytokinin decrease during prolonged cold treatment (final sampling point) might 16 reflect transition from acclimation to maintenance response phase (Larcher, 2003). Similarity 17 of the response of cytokinin phosphates in both genotypes might be given by the prevailing 18 effect of low temperature that negatively affects cytokinin biosynthesis and, as a consequence, 19 plant growth rate.

20 The concentration of active cytokinins is controlled at transcriptional level by several 21 isopentenyl multigene families including transferases for synthesis, cytokinin 22 oxidases/dehydrogenases for degradation, trans/cis-zeatin O-glycosyltransferases for 23 reversible inactivation and beta-glucosidases for reactivation (Song et al., 2012). Among 24 them, a gene encoding zeatin O-glycosyltransferase was affected by the mvp2 mutation and 25 corresponding differences were found in *cis/trans*-zeatin O-glucoside content between wt and 26 mvp2 plants. Although we did not find such differences in the expression of other genes 27 related to zeatin metabolism by transcript profiling, a coordinated regulation of these genes 28 was observed during the reproductive development of maize in a previous study. It is possible 29 to assume that differences in cytokinin related transcripts have not been detected in our study 30 due to their relatively fine regulations, their subtle changes being below our threshold limit.

31 9 Conclusions

32 Comparison of wild type and *mvp2* deletion mutant wheat plants showed that the deleted 33 region regulates a whole set of genes affecting freezing tolerance and vegetative/generative transition. Among these genes also occur those ones that are related to SA and cytokinin metabolism, and may be responsible for the differences in the level of these hormones between the wild type and mutant plants. Thus, the present results indicate the involvement of these plant hormones in the VRN1-dependent control of flowering. Our results also contribute to the better understanding of the link between the stress responses and developmental processes in wheat plants.

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1 **12 Figure legends**

2 Figure 1.: Morphology of the shoot apices during vegetative/generative transition. The 3 following developmental stages are shown: vegetative phase before the start of cold treatment (growth at 20/17 °C) (VP 20 °C), vegetative phase at 4 °C (VP 4 °C), double ridge stage (DR) 4 5 and initiation of spike primordia (SI). Only the apices of Triticum monococcum wt showed these phenophase changes. The morphology of *mvp2* mutant apices remained very similar 6 7 during the treatment – aside from a short elongation – because this genotype cannot flower. 8 The four sampling points were chosen according the wt development, but the same 9 nomenclature was used in case of the mutant genotype. The white bars indicate 100 µm.

10

Figure 2.: Set diagram of the genes which exhibited higher than 2 fold-change of expression
in relation to the genotype (G), developmental phase (D) or their interaction (D × G).
(According to two way ANOVA.)

14

15 Figure 3.: Relative expression of the genes used for the validation of the microarray results. 16 A - CBF4 (C repeat-binding factor4); B - CBF14 (C repeat-binding factor14); C - R2R3-MYB (R2R3-MYB protein); D - VRN1 (vernalization 1); E - C2H2 (C2H2 Zn finger protein); F - STPK 17 18 (serine-threonine protein kinase); G - LG (lipoxygenase); H - HSP80 (heat shock protein 80 KDa); 19 I - HSP101 (heat shock protein 101 KDa); J - HSP17 (small heat shock protein 17.3 KDa); K -20 MPBF1 (multiprotein bridging factor 1); L - PAL (phenylalanine ammonia-lyase). All the 21 expression values were compared to those detected in wt at DR phase. $\Delta\Delta C_t$ method was used for 22 the data analysis. All y-axes show the relative normalized expression at log₂ scale. The error 23 bars show the standard deviation.

24

Figure 4.: Filtered heat-map of the selected genes differentially expressed in *mvp2* and wt plants

- at the DR phase, which exhibited expression difference higher than 2 or smaller than 0.5.
- 27

1 Figure 5.: Networks of interacting proteins. The main coding genes (AP1, SEP4, SAG12 and 2 PHYC, i.e., the homologs of the genes deleted in the mvp2 mutant: VRN1, AGLG1, CYS and 3 PHY-C, respectively) are indicated by deep blue circles. Genes that were not represented in 4 the array experiment are indicated by light blue and smaller circles. All other genes received a 5 special three-parted specification. Left sector represents the relative expression of *mvp2* at DR phase compared to wt at DR. Right sector represents the expression of wt at SI phase 6 7 compared to wt at DR. The bottom sector represents the expression of mvp2 at SI phase 8 compared to wt at DR. Direct connections are shown with red lines, while the indirect 9 connections with orange and black lines.

10

Figure 6.: Salicylic acid biosynthesis and the changes in the crowns and leaves of the examined plants. Accumulation of bound and free 2-hydroxy-cinnamic acid (*o*HCA) in crowns (A) and leaves (B), of bound and free cinnamate (CA) in crowns (C) and leaves (D), of bound and free salicylic acid (SA) in crowns (E) and leaves (F) and of bound and free benzoate (BA) in crowns (G) and leaves (H).

16

Figure 7.: Comparison of cytokinin content in the leaves of *mvp2* mutant and wt during the
vegetative/generative transition at 4 °C. A – cytokinin metabolites down-regulated in *mvp2*mutant; B - cytokinin metabolites up-regulated in *mvp2* mutant.

20

Supplemental Figure 1: Regression analysis between micro-array and qRT-PCR. Relative fold changes in *mvp2* at DR, *mvp2* at SI, wt at DR and wt at SI. In every case expression values were compared to wt at DR. 'x' coordinates show the result in micro-array, 'y' coordinates show the results in qRT-PCR.

25

Supplemental Figure 2: Expression pattern of genes that exhibit more than 2-fold difference (on log₂ scale) in the expression rate of genotypes and/or developmental stages. In the first column, two developmental phases (DR and SI) of wt were compared to each other; in the second and third columns, SI and DR phases of *mvp2* was compared to the DR phase of wt. These 200 genes represent well-annotated, non-redundant wheat proteins with E-value \leq 1e-40.

1 Supplemental Figure 3: Result of gene ontology analysis according to biological processes. 2 The diagram shows the number of the genes associated with the specified process. In the right 3 bottom of the figure the set-diagram represents the clusters of pathways in connection with 4 the genotype (G), developmental phase (D) and both $(D \times G-D-G)$. 5 6 Supplemental Figure 4: Result of gene ontology analysis according to molecular functions. 7 The diagram shows the number of the genes associated with the specified functions. In the 8 right bottom of the figure the set-diagram represents the clusters of pathways in connection 9 with the genotype (G), developmental phase (D) and both $(D \times G-D-G)$. 10 11 Supplemental Figure 5: Clusterisation of the genes in connection with KEGG pathways. In 12 the right bottom of the figure the set-diagram represents the clusters of pathways. 13 14 Supplemental Figure 6: Zeatin biosynthesis according to the KEGG database. All of the 15 measured cytokinins are marked with blue circles. 16 17 Supplemental Figure 7: Cytokinin content in the leaves of the examined plants during the 18 vegetative/generative transition at 4 °C. Cytokinin metabolites were grouped according to 19 molecular structure and biological activity. A - cytokinin bases ; B - cytokinin ribosides; C -20 cytokinin monophosphates; D – cytokinin N-glucosides; E – cytokinin O-glucosides. 21 22 Supplemental Table 1: Primers sequences.

1 2: Supplemental Table Genes selected for the analyses. 2 Row A represents the selected genes whose expression changes were visualized on the heat-3 maps (Fig. 4 and Supp. Fig. 2). The full heat-map (Supp. Fig. 2) represents 200 genes (A/5). 4 Only those were selected for the reduced map (Fig. 4) that exhibited differences between the 5 at DR phase smaller than 0.5 or 2 genotypes bigger than (A/6). 6 Row B presents the dataset selection for gene ontology analysis. In B/2 cells numbers of those 7 genes are indicated where the changes between the compared groups were higher than or 8 equal to 2 [D: according to development phase (DR and SI); G: according to genotype (mvp2 9 and wt) and D × G-D-G: according to common set of D, G and D × G] and the p-value ≤ 0.05 . Arabidopsis homologs were selected based on this dataset if the E-value \leq 1e-40 (B/3). Only 10 those expression results related to the same genes were kept that exhibited the best E-values 11 12 (B/4). The selected genes were analysed by BiNGO Cytoscape plugin software to determine 13 over-represented genes connected to biological processes (B/5) and molecular functions (B/6). 14 For the functional annotation of genes, KEGG database was used (B/7). 15 Row C shows the selected dataset of those genes that were visualized in Fig. 5 showing networks of interacting proteins. In the end, 59 genes (Arabidopsis homologs) were identified 16 17 in the BioGrid network.





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Figure6 Click here to download high resolution image



Figure7 Click here to download high resolution image







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Suppl.Figure2

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	A 99 _P140538 gbjABK55379.1 (CBFIVb-D21 [T. ae.] [3E-98] A 99 _P317441 gbjABK55385.1 (CBFIVd-9.1 [T. ae.] [1E-111] A _99 _P079875 gbjABK55387.1 (CBFIVd-9 [T. ae.] [1E-102]
	A 99 P074325 gblABK55383.1 [CBFIVd-4.1 [T. ae.] [1E-122] A 99 P079820 gblAAT76662.1 [1 aCBF14(C repeat-binding factor 2 [T. ae.] [7E-147] A 99 P105175 refINP 176927.1] acid phosphataset/-dependent haloperoxidase-related protein [A. th.] [5E-7] A 90 P103575 gblABK55321 [LCBFIVA 42 [T. ae.] (HE 132]
6	A 99 - P278321 emb(CAE53900.1] putative MADS box-like protein [T. ae.] [1E-102] A 99 - P278321 emb(CAE53900.1] putative MADS box-like protein [T. ae.] [1E-102] A 99 - P229586 gb]ACC90366.1] perxidase precursor [T. ae.] [6E-41] A 99 - P083870 uplABK55360.1] CBFILIA D6 [T. ae.] [4E-106]
	A 99 P000681 jblAAX28964 1 TaCBF6 [T. ae.] [2E-97] A 99 P641396 gblAAU08785 1 NAC domain transcription factor [T. ae.] [4E-53] A 99 P429112 ref(NP 181527 1] HXXXD-type acyl-transferase-like protein [A. th.] [3E-76]
	A 99 -P234046 gb/AL757684.1 neavy metal transporting P1B-ALP382 [1. ae.] [45-46] A 99 -P225531 gb/ACF08082.1 class III peroxidase [1. ae.] [47-175] A 99 -P086620 refINP 179361.11 ACT-Ilike protein tyrosine kinase-like protein [A. th.] [5E-40] A 99 -P577077 ubilsAF02546 11 triticain joha [1. ae.] 14E-1541
ſ	A_99_P636061 emb CAA04543.1 sucrose synthase type I [T. ae.] [3E-162] A_99_P162767 refNP_567936.5 fatty acyl-CoA reductase CER4 [A. th.] [4E-61] A_99_P250636 dbj BAF51565.1 galactinio synthase [T. ae.] [5E-156]
	A 99 P550797 refINP 567835.1 (CFIM-25-like protein [A. th.] [3E-46] A 99 P247391 refINP 181372.1 peroxidase 22 [A. th] [1E-45] A 99 P493777 refINP 193139.1 selenium-binding protein 1 [A. th.] [1E-49] A 99 P1291349 refINP 001185033.1 [P40MB01D.like protein 1 [A. th.] [1E-63]
	A_99_P263521 dbjlBAM74038.1] cell will invertase [T. ae.] [1E-116] A_99_P24759 spl(22UXF7.1] Fructan 6-exohydrolase [T. ae.] [8E-103] A_99_P348596 emb(CA.1933.4.1] UDP-glucose glucose ylucosyltransferase [T. ae.] [1E-103]
	A 99 P420007 gb AAC19321 1 chalcone synthäse [T. ae.] [8E-165] A 99 P431722 emb(CAP72304 1 C-methyltransferase [T. ae.] [6E-102] A 99 P334001 ret[NP 196153 1] peroxidase 52 [A. th.] [9E-71]
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	A 99 _570947 g0fA4000597 11 sucross-phosphate synthase 9 [T. ae.] [1E-96] A 99 _P535882 gblAAQ10452.1 sucross-phosphate synthase 9 [T. ae.] [1E-96] A 99 _P234356 gblAAS49905.2 Jubative proteinase inhibitor-related protein [T. ae.] [2E-50] A 99 _P453392 erlNP 18059.2 J biogenesis of tysosome-related organelles complex 1 subunit 1 [A. th.] I7E-4
	A _99_P241881 dbjlpA/N63108.1] wheat cold induced 16 [T. ac.] [1E-52] A _99_P249236 emb(CAH69190.1] type 1 non specific lipid transfer protein precursor [T. ac.] [2E-42] A _99_P584562 splP46526.1] Cold shock protein CS66 [T. ac.] [2E-46]
	A 99 -P300212 relivity 505953.2 [priospholipase Dibera 1 [A. th.] [bt=-52] A 99 -P229571 relivity = 76102 relivity and a chydrogenase family protein [A. th.] [2E-85] A 99 -P466377 dbi[BAC0112 1] LEA D-11 dehydrin [T. ae.] [4E-60] A 99 -P240651 dbi[BAC0142 1] drom 21 drom 21 dre phylogenesis abundant protein [T. ae.] [2E-52]
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	A 99 _= F10315 gblAA67001.1 defensin [T. ae.] [Z=-53] A 99 _= P261372 gblAA67001.1 defensin [T. ae.] [Z=-53] A 99 _= P222301 gblA2614253.1 [TCER1 [T. ae.] [6E-54] A 99 = P637881 gblA281542 1 ice recrystallization inhibition protein 1 precursor [T. ae.] [Z=-95]
	A_99_P305251 gb/ABB02411.11 chloroplast lipocalin [T. ae.] [1E-88] A_99_P413582 gb/ADG85703.11 NAC transcription factor [T. ae.] [6E-70] A_99_P387057 refNP_188499.11 AAA-type ATPase family protein [A th.] [6E-42]
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	A 99 - P270326 09/HP07942-11 (WADS-DOX trainschiption 1406142 [1]: 4. [1] [2:5-2] A 99 - P439652 fell/P 192948. 1] sterol-4alpha-methyl oxidase 1-1 [A th.] [1:E-54] A 99 - P175019 db][BA776309.1] early light-inducible protein [T. ae.] [5E-64] A 99 - P174189 ob]AAX132611 1] ethylene-responsive element binding protein 2 [T. ae.] [3E-78]
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	A 99 P000481 gblAAD22629.1 heat shock protein 101 [T. ae.] [3E-60] A 99 P428097 gblA2Y83978.1 small heat shock protein 17.3 KDa. partial [T. ae.] [1E-93] A 99 P543487 gblAAD03604.1 small heat shock protein Hsp23.5 [T. ae.] [2E-61] A 90 P029206 gblACU4263.1 smillineatic bedrage for the 2.5 [1. 36.] [2E-61]
	A 99 P449692 PfNP 190209.1] heat shock protein 17.4 [A. th.] [3E-40] A 99 P449692 FiNP 190209.1] heat shock protein 17.4 [A. th.] [3E-40] A 99 P4482257 sp[F8RP11.1] Hsp70-Hsp90 organizing protein [T. ae] [3E-40] A 99 P244661 refNP 196763.1] class II heat shock protein 17.6 [A. th.] [3E-53]
f	A_99_P324301 refNP_180337.1 ovtochrome P450, f 94, sf C, pp 1 [A. th.] [1E-52] A_99_P072890 gb/ABG21009.1] MADS2 [T. ae.] [8E-84] A_99_P389287 refNP_187923.1] meiotic recombination protein SPO11-1 [A. th.] [3E-66]
(A 99 -P009801 gD(AFP49521.1) etmylene responsive transcription factor 6, partial [1, ae,] (ae-40) A 99 -P315231 etfN(P 193368.1) maintenance of ploidy protein MOB1 (A th.] [1E-114] A 99 -P244361 etmb(CAA68913.1) peptidy/proly isomerase [T, ae,] (3E-120) A 99 -P338406 etml(P 172961.1) lind hosenbate sponshatase 2 (4 th.) (1E-53)
	A 99 P290131 reflyP 173043.11 factor of DNA methylation 1 [A. th.] [4E-55] A 99 P374667 reflyP 566892.11 putative LRR receptor-like serine/threonine-protein kinase [A. th.] [5E-62] A 99 P163217 oplAAR51797.11 small heat shock protein HSP17.8 [T. ae.] [5E-81]
	A 99 P036254 embjCAD30692 11 fatty acyl coA reductase [T. ae.] [2E-95] A 99 P400502 g0jAlV25733 11 heat shock factor A6 [T. ae.] [3E-109] A 99 P477327 ref[NP_567456 1] extradiol ring-cleavage dioxygenase [A, th.] [6E-80]
	A 99 P402297 gD4AC49255 1 lipoxyganase partial [T. ae.] [1E-93] A 99 P138430 gb1AC49255 1 lipoxyganase, partial [T. ae.] [1E-93] A 99 P359066 ref[NP 973799 1] Respiratory burst oxidase B [A. th.] [8E-53] A 99 P274716 ref[NP 1870791 1] Licioerant lipase 1 (A. th.) [1E-42]
j i i i i i i i i i i i i i i i i i i i	A_99_P066330 ref(HP_196179.1] oxidoreductase, 2OG-Fe(II) oxygénase family protein [A. th.] [3E-71] A_99_P218071 gb/AFK30379.1] small heat shock protein [T. ae] [1E-80] A_99_P213261 gb/ABI96817.1] tonoplast intrinsic protein [T. ae] [5E-76]
	A 99 P360546 refINP 182038.1 [SPX domain protein 3 [A. th.] [4E-44] A 99 P016799 p0 AAUS0665.1 ADP-glucose pryophosphorylase small subunit [T. ae.] [5E-54] A 99 P268781 gb AAP81746.1 adenine phosphoribosyltransferase [T. ae.] [3E-105] A 99 P260365 epiAAV84679.1 U/AE email eubruit (T. ae.) [47 44]
	A 99 – 200305 gl/ADF31759.1 Hsp90.2-D1; heat shock protein 90 (T. ae.) [4E-112] A 99 – P00366 gb/ADF31759.1 Hsp90.2-D1; heat shock protein 80 KDa, partial [T. ae.] [4E-112] A 99 – P000366 gb/AE783982.1 heat shock protein 80 KDa, partial [T. ae.] [1E-102] A 99 – P412732 gb/ABU95041.11 (C2H2 Cm finger protein IT. ae.] [6E-57]
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	A_99_P2448647 gb/AEO22185.1 CytHSP70 [T. ae.] (6E-83) A_99_P278026 gb/AEO22185.1 CytHSP40, partial [T. ae.] (3E-61) A_99_P224731 eftyNP_850984.4 Heat shock protein 70 [A. th.] [TE-55]
	A 99 P223831 gb AAD03605.1] small heat shock protein Hsp23.6 [T. ae.] [3E-106] A 99 P302836 erl(NP_201068.1] putative beta-1.3-galactosytransferase (A. th.] [3E-103] A 99 P149593 gb AAC23502.1] vacuolar invertase (T. ae.] [3E-41] A 99 P149593 gb AAC23502
1 	A 99 – P320336 dbjlEAN81752.1 ABA 8-hydroxylase [1 ae,] [5E-50] A 99 – P320336 dbjlEAN81752.1 ABA 8-hydroxylase [1 ae] [5E-110] A 99 – P347746 gb]AEV91184.1 MYB-related protein [T. ae] [7E-43] A 99 – P220571 emD(CAZF0654 1) glyceraldehyde-3-bhosphate dehydrogenase [T. ae,] [4E-121]
	A_99_P316936 ref(NP_195029.1] sulfoquinovosyldiacylgiverol 1 [A. th.] [2E-56] A_99_P161637 gb]AAS07016.1] S-like RNase [[T. ae.] [5E-103] A_99_P457732 ref(NP_199851.2] purple acid phosphatase 27 [A. th.] [2E-63]
	A 99_P337001 refINP_189020.21 nuclear RNA polymerase D2A [A. th.] [3E-87] A 99_P233346 refINP_193470.1 60S ribosomal protein L15-2 [A. th.] [7E-59] A 99_P231496 refINP_181256.11 ribosomal protein L11 family protein [A. th.] [4E-101] A 99_P26431 refINP_181256.11 ribosomal protein L11 family protein [A. th.] [4E-101]
4	A 99 P31936 relivP 56603.11 ribosomal protein 52/r3516.4 http://t-5/j A 99 P31938 relivP 56603.11 ribosomal protein 52/r3516.4 http://t-5/j A 99 P312346 relivP 182320.11 transducin family nucleolar protein with WD40 repeats [A. th.] [1E-53] A 99 P312346 relivP 186994.21 RM repeat superfamily protein (A th 118-451)
	A_99_P255071 gb/AFV51839.1[famesyl pyrophosphate synthase [1, ae.] [3E-143] A_99_P254361 gb/ASU41846.151 A_99_P009461 refNP_001185207.1[ubiquithr-conjugating enzyme family protein [A. th.] [2E-89]
	A 99. P169904 sp(384VQ1 1] SNF1:elated protein kinasë regulatory subunit beta-1 [A fth] [7E-40] A 99. P522721 refNP 974592.1] ovysterol binding protein-related protein 2A [A fth.] [4E-65] A 99. P561567 gb(A&R11387.1] ovtochrome P450 [T. ae.] [2E-75]
	 A 99 P054905 pprocessor in loncent endor i, o-beta-glucosidase (1, ae.) (bE-14/) A 99 P15602 effNP 19972.11 methionine S-methylitransferase (A, th.) [1E-56] A 99 P159149 refINP 7001154652 2) endoribonuclease Dicertlike 3 [A, th.] [2E-61] A 99 P294221 refINP 568171 11 mitochondrial substate carrier family protein (A, th.) [15E-58]
	A_99_P129195 dbjlBAE80455 1j Tamyb2, MYB-related protein (T. ae. [4E-50] A_99_P031129 gb]AAY84973 1j Tamyb2, MYB-related protein (T. ae. [4E-50] A_99_P281196 eft]NP_565518, il casein lytic proteinase B4 [A, th] [3E-46]
	A_99_P438252 gb AFK26595.1 inorganic pyrophosphotase [T. ac.] [5E-61] A_99_P158527 refNP_567881.1 RNA Lariat debranching enzyme [A. th.] [4E-117] A_99_P471152 gb ADF31758.1 Hsp90_2-B1: heat shock protein 90 (T. ac.) [5E-151]

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 Con 22847 gb)AFX26505 11, seriner/threonine protein kinase Stpk/B [T. ae] (2E-74)
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 Con 22847 gb)AFX26505 11, seriner/threonine protein phosphatase 2C 27 [A. th] (2E-103)
 Con 22847 gb)AFX26505 11, WRXY14 transcription factor [T. ae] (1E-179)
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Suppl.Figure3 Click here to download high resolution image



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Zeatin biosynthesis

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Name	Function	Forward	Reverse	Amplicon length	Reference	GeneID
mvp-CBF4	C repeat-binding factor4	GGCGGGGGGGGAGGATCAAGTACAA	GAATCGGCGAAGTTGAGGCAG	222 bp		ABK55379.1
mvp-CBF14	C repeat-binding factor14	AAGGAGATCAAGGACGCCGTC	CCGAGTAGAACGATCCGGCAT	208 bp		AAT76662.1
mvp-STPK	serine-threonine protein kinase	CTCCGTTCGGTCCGTGAATCT	GCCCGAGCCTATGTCCTTGAT	157 bp		AIK01699.1
mvp-R2R3MYB	R2R3-MYB protein	TTGGTCGTCGATCCTCCACAG	GAGGAGAAGAAGGCGGAGGTG	210 bp		AEV91144.1
mvp-LG	lipoxygenase	TGGGCCAGCAAAATTCCCCTA	GCCACTCATGAAACGTGCACA	195 bp		AAC49285.1
mvp-C2H2	C2H2 Zn finger protein	TCGATGCAGATCCACCACGAG	TACCCCGTCTTCTTGAGGCTG	164 bp		ABU95041.1
mvp-HSP17	heat shock protein 17.3 KDa	CATCAAGGTGCAGGTGGAGGA	CCTGGATGGTCTTGGGCTTCT	242 bp		X58279.1
mvp-MPBF1	multiprotein bridging factor 1	GACGAACAAGAATGCCTCCGC	CTCTCGTACTCCTGCACCACC	201 bp		GQ370008.1
mvp-HSP80	heat shock protein 80 KDa	TTCCAGGCCGAGATCAACCAG	GTGTGTTCGTGGCCTTGTCAG	202 bp		AEY83982.1
mvp-HSP101	heat shock protein 101 KDa	GGTGACGCAGCTGTCCAAGAT	AGGATGTCGGACCTCTGCCC	153 bp		AAD22629.1
VRN-1		ACAAGAAAAACACTTGCAGAGAAGTTCAGC	CATGGTAAATTACTCGTACAGCCATCTCAGC	1084 bp	(Juhász et al. 2015)	AY747599.1
mvp F18 and mvp R22	MADS-box	AGCCACAAGAACCGGGACTA	ATTCAAGCCCCAATGTTCTC	172 bp (Dhillon et a		AY747599.1
mvp F18 and mvp R23	factor	AGCCACAAGAACCGGGACTA	CCCAAACTTTGCGGTGTATC	339 bp	2010)	AY747599.1
Ex4-5 and Ex8_R1-ABD		TCAGATCCAGGAAGAACCAA	TTGATGTGGCT M ACCATCCA	313 bp (Loukoianov et al. 2005)		AY747599.1
Ta30797		GCCGTGTCCATGCCAGTG	TTAGCCTGAACCACCTGTGC	126 bp	(Paolacci et al. 2009)	TC279294
TaPAL	phenylalanine ammonia-lyase	GCGGTCTCCTGGCGAAGAGG	GCACACTACTCTTCACTTTCTCGATCC	173 bp		HX081317

Supplemental Table 2.

	1	2	3	4	5	6	7
А		Σ	WHEAT or ARATH 1e-40	WHEAT or ARATH 1e-40 Non- Redundant	Full HEATMAP	Filtered HEATMAP	
	Full datasets	1064	319/1064	200/1064	200/1064	137/1064	
		Σ	Arabidopsis 1e-40	Arabidopsis 1e-40 Non- Redundant	Gene Ontology Biol. Proc.	Gene Ontology Mol. Funct.	Genes in KEGG Pathways
В	D	198	45	29	20	26	14
	G	306	56	49	40	48	21
	D×G-D-G	237	46	29	25	26	9
С		Σ	Arabidopsis annotation	Arabidopsis annotation + TAIR ID	TAIR ID in Network	TAIR ID in Network 1e-40	
	Full datasets	1064	796/1064	790/1064	59/9264	16/9264	