Redox control of miRNAs and their targets in wheat

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Highlight

A redox-dependent regulatory network of miRNAs and their targets were created using sequencing results, bioinformatics tools and correlation analysis of the examined biochemical and molecular parameters in wheat.

Abstract

Possible redox control of miRNAs was investigated in wheat. One-day treatment of seedlings with 10 mM H_2O_2 resulted in decreased glutathione content and increased half-cell reduction potential of the glutathione disulphide/glutathione redox pair and greater ascorbate peroxidase activity compared to the control plants. These changes were accompanied by alterations in the miRNA transcript profile, since 70 miRNAs with at least 1.5-fold difference in their expression between control and treated (0, 3, 6 h) seedlings were identified. Their 86 target genes were determined by degradome sequencing and 6808 possible additional target genes were identified by bioinformatics tools. The H_2O_2-responsiveness (24 h treatment) of 1647 targets was also confirmed by transcriptome analysis. They are mainly related to the control of redox processes, transcription and protein phosphorylation and degradation. In a time-course experiment (0, 1, 3, 6, 9, 12, 24 h treatment) a correlation was found between the levels of glutathione, other antioxidants and the transcript levels of the H_2O_2-responsive miRNAs and their target mRNAs. This relationship together with the bioinformatics modelling of the regulatory network indicate the glutathione-related redox control of miRNAs and their targets, which allows the adjustment of the metabolism to changing environmental conditions.

Key words: Ascorbate, glutathione, hydrogen-peroxide, miRNAs, redox regulation, wheat.

Abbreviations: AsA: ascorbic acid, APX: ascorbate peroxidase, CAT: catalase, GR: glutathione reductase, GSH: glutathione, GSSG: glutathione disulphide, GST: glutathione S-transferase, ROS: Reactive oxygen species.

Introduction

Reactive oxygen species (ROS) and antioxidants have an important role in the regulation of growth and development both under optimal and stress conditions (Kocsy *et al.*, 2013; Considine and Foyer, 2014; Locato *et al.*, 2018). Among ROS, H_2O_2 is the most stable ROS, which makes it appropriate for long distance signalling and controlling of various metabolic processes at the level of gene expression and protein activity (Foyer *et al.*, 1997; Neill *et al.*, 2002; Hossain *et al.*, 2015). Consistent with this hypothesis, increased H_2O_2 in catalase-deficient *Arabidopsis thaliana* (L.) Heynh. mutants resulted in the induction of genes related to the regulation of stress response, metabolism, development and energy homeostasis (Vandenabeele *et al.*, 2004). In H_2O_2-treated *Arabidopsis*, altered expression of genes encoding proteins involved in the transcription, signal transduction, protein transport, energy homeostasis, cellular organisation and defence processes was observed by microarray analysis (Desikan *et al.*, 2001). Similarly to *Arabidopsis*, genes involved in cell defence, signal transduction and metabolism (carbohydrates and lipids) were also affected by H_2O_2 treatment in wheat (Li *et al.*, 2011). Besides these genes, the expression of redox homeostasis- and photosynthesis-related genes also changed in wheat. By a proteomic approach, the effect of exogenous H_2O_2 on most of these processes was also shown in rice (Wan and Liu, 2008). Besides its effect on the total amount of proteins, H_2O_2 may also regulate their activity due to the oxidation of the Cys residues. The amount of H_2O_2 is regulated by the ascorbate-glutathione (AsA-GSH) cycle which is composed of both enzymatic and non-enzymatic components affecting indirectly the H_2O_2-dependent physiological processes (Roach *et al.*, 2018). Recently, the direct regulatory role of AsA and GSH was shown to modify the development of reproductive organs and tolerance to low temperature through their effect on redox system and gene expression (Gulyás *et al.*, 2014).

Similarly to the AsA-GSH cycle, microRNAs (miRNAs) are also important in the control of development (Kidner and Martienssen, 2005; Rubio-Somoza and Weigel, 2011) and stress response (Phillips *et al.*, 2007; Khraiwesh *et al.*, 2012; Rajwanshi *et al.*, 2014). They can control the expression level of their target genes transcriptionally by DNA methylation and post-transcriptionally by cleavage or translational inhibition of target mRNAs. Many of the target genes of miRNAs encode transcription factors; therefore, one miRNA is able to regulate indirectly a whole set of genes. miRNAs have a pleiotropic effect in the control of development, but one target gene may also be regulated by more miRNAs (Kidner and Martienssen, 2005). They are components of regulatory networks that coordinate

gene expression programs ensuring developmental plasticity (Rubio-Somoza and Weigel, 2011). Such network was described for developmental phase transitions, leaf senescence, cell proliferation and leaf polarity; furthermore the interconnection of miRNA-dependent regulatory networks has also been suggested under both biotic and abiotic stresses (Rajwanshi *et al.*, 2014). In wheat, many miRNAs related to development and stress response were discovered (Yao and Sun, 2012) either by computational approach (Dryanova *et al.*, 2008) or by next generation sequencing (Sun *et al.*, 2014). Although miRNAs are evolutionarily conserved, several monocot- or wheat-specific miRNAs were described. By surveying miRNA profile in 11 different tissues, 323 novel miRNAs (belonging to 276 families) and 524 targets for 124 miRNAs were identified in a recent study of wheat (Sun *et al.*, 2014). When the redox regulation of miRNAs was studied in rice, 7 H_2O_2-responsive miRNAs were identified that are involved in transcriptional regulation, nutrient transport, auxin homeostasis, cell proliferation and programmed cell death (Li *et al.*, 2011). In *Brachypodium distachyon* (L.) P. Beauv. 61 H_2O_2-responsive miRNAs were determined the target of which were related to development, reproduction, response to stress, secondary metabolism, catabolic processes, nucleic acid metabolism and cellular component organization (Lv *et al.*, 2016).

The aim of the present study was to find out whether the effect of H_2O_2-induced oxidative stress on miRNAs and their target genes is mediated by the GSH and other antioxidants in wheat. For these purpose the determination of the H_2O_2-reponsive miRNA and target mRNA profiles and regulatory networks were planned. The proposed relationship between the various antioxidants and the levels of several miRNAs and their targets were checked in a time course experiment.

Materials and methods

Plant material and treatments

Seeds of the wheat variety *Triticum aestivum* L. ssp. *aestivum* cv. *Chinese Spring* (CS) were germinated in Petri dishes (1 day at 25°C, 3 days at 4°C, 2 days at 25°C). Seedlings were grown on half-strength modified Hoagland solution with a photoperiod of 16 h, at 260 µmol m^{-2} s^{-1}, 22 °C and 75% RH in a growth chamber (Conviron PGV-15; Controlled Env., Ltd., Winnipeg, Canada) (Kocsy *et al.*, 2000). After 10 days of growth (2-leaf developmental stage), 10 mM H_2O_2 was added to the nutrient solution and sampling was done after 0- (8 h light), 1- (9 h light), 3- (11 h light), 6- (14 h light), 9- (1 h dark), 12- (4 h dark) and 24-hour (8 h light) treatments. The first sampling of leaves was performed in the middle of the 16-hour

light period in order to exclude the possible rapid changes that might occur in certain examined parameters just after switching on the light. Besides the collection of leaf samples (2^{nd} leaves from the basis of the stem) for biochemical and molecular biological analysis, the fresh and dry weights of the shoots and roots were also determined.

Measurement of H_2O_2 content

H_2O_2 content of the leaves was measured by FOX1 method using a spectrophotometer in a colorimetric reaction as described in a previous study (Kellős et al., 2008). During this reaction ferrous ion is oxidised to ferric ion by H_2O_2 and the ferric ion is detected by xylenol orange.

Determination of AsA

Leaf samples of 500 mg fresh weight were ground with liquid nitrogen in a mortar and extracted with 3 ml of 5% meta-phosphoric acid. In the supernatant, reduced and total AsA (the latter reduced by dithiothreitol) contents were determined by HPLC using an Alliance 2690 system equipped with a W996 photodiode array detector (Waters, Milford, MA, USA). The concentration of dehydroascorbate (DHA), a two-electron oxidized form of AsA was estimated by subtracting the reduced portion from the total AsA pool (Szalai *et al.*, 2014).

Analysis of cysteine and glutathione

The leaves were ground with liquid nitrogen in a mortar, after which 1ml of 0.1 M HCl was added to 200 mg plant sample. The total cysteine and glutathione pools (reduced + oxidised forms) were determined after reduction with dithiothreitol and derivatisation with monobromobimane (Kocsy *et al.*, 2000). For the detection of cystine and glutathione disulphide (GSSG), cysteine and GSH were blocked with N-ethylmaleimide, after which the excess of N-ethylmaleimide was removed with toluol (Kranner and Grill, 1996). Cystine and GSSG were reduced and derivatised as described for total cysteine and glutathione pools. The two thiols were analysed by an Alliance 2690 HPLC system using a W474 scanning fluorescence detector (Waters, Milford, MA, USA). The amount of reduced thiols was

calculated as the difference between the amount of total and oxidised thiols. The half-cell reduction potential of the thiol redox couples was calculated by the Nernst equation (Schafer and Buettner, 2001).

Analysis of the activity of the antioxidant enzymes

The activities of catalase (CAT, EC 1.11.1.6; basis: reduction of H_2O_2), ascorbate peroxidase (APX, EC 1.11.1.11; basis: reduction of H_2O_2 by AsA), glutathione reductase (GR, EC 1.6.4.2; basis: reduction of GSSG by NADPH and reaction of the produced GSH with 5-5″-dithio- bis (2-nitrobenzoic acid)) and glutathione S-transferase (GST, EC 2.5.1.18; basis: reaction of GSH with 1-chloro-2,4-dinitrobenzene) were determined in the leaves by spectrophotometer using a colorimetric method as described previously (Soltész *et al.*, 2011). The extraction buffer contained 1 mM AsA in order to avoid the inactivation of chloroplastic APX as suggested by Noctor *et al.* (2016).The protein content was measured using Bradford-reagent (Bradford, 1976) according to Soltész *et al.*, (2011).

Small RNA sequencing

Total RNA was isolated from the leaves of plants treated for 0-, 3- and 6-h with 10 mM H_2O_2 using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. All small RNA libraries prepared for this study according to Sun et al. (2014) were sequenced with Illumina Hiseq 2000, generating approximately 10 M data for each sample. Low-quality reads and portions of reads were removed using sickle program (https://github.com/najoshi/sickle) with the parameters "-q 20 -f sanger -l 20". The cutadapt program (Martin, 2011) was used to trim the 3' adaptors from reads (parameters " -CTGTAGGCACCATCAATCAG - match-read-wildcards -m15 "), and only the reads ranging from 18 to 30 nucleotides were collected. Reads were then aligned to the Rfam 10.0 RNA family database (Griffiths-Jones *et al.*, 2003; Griffiths-Jones, 2004; Nawrocki *et al.*, 2015) with bowtie2 (Langmead and Salzberg, 2012) and known cellular structural RNAs, such as rRNAs, tRNAs, snoRNAs, and snRNAs were removed based on their alignments using in-house Perl script. The remaining reads were mapped to wheat microRNAs (Sun *et al.*, 2014) collected and characterised by BLASTN and allowing no mismatches. The miRNA frequency

was normalised as "transcripts per million" (TPM), and the expression was set to 0.01 for miRNAs that were not expressed in one of the samples after normalisation.

Following the first filtering for reliability, the differentially expressed miRNAs were obtained by Bayes-based Poisson Distribution Test (Audic and Claverie, 1997) with difference >1.5 times and sequencing reads >10 in at least one sample.

Analysis of miRNAs by qRT-PCR

Total RNA was isolated from frozen leaves using Trizol (Invitrogen, USA). The Mir-X miRNA First-Strand Synthesis Kit (Clontech Laboratories, Inc) and SYBR Premix EX Taq II (TaKaRa, Dalian, China) were used for miRNA reverse transcription and qRT-PCR according to the manufacturer's instructions. qRT-PCR was performed on the CFX96 Real Time System (BIO-RAD, USA) with the following program: denaturation at 95°C for 3 min, and then subjected to 40 cycles of 95°C for 15 s, 60°C for 15 s, 72°C for 10 s. The entire sequence of the mature miRNA (21–23 nt) was used as a miRNA-specific 5' primer. The 3' primer for the qPCR was the mRQ 3' primer supplied with the kit. The relative expression of miRNA was calculated using the $2^{-\Delta CT}$ method normalised to wheat *ACTIN* gene CT value. For each sample, the PCR amplification was repeated three times, and the average values of $2^{-\Delta CT}$ were used to determine the differences of gene expression by the Student's *t*-test. Three biological replications were performed with similar results and one replicate was shown.

Degradome sequencing

Total RNAs obtained from leaves taken after 0 h, 3 h and 6 h H_2O_2 treatment were mixed equally for degradome sequencing to see which miRNA targets are cleaved at any time point. The degradome library was constructed briefly as followed: annealing of approximately 150 ng poly(A)-enriched RNA with Biotinylated Random Primers; Strapavidin capture of RNA fragments through Biotinylated Random Primers; 5′PARE adaptor ligation to only those containing 5-monophosphates; first-strand cDNA was generated from the ligated sequence after reverse transcription using random hexamer 3′ primer; a number of DNA products were produced by PCR amplification. The library was single-end sequenced using an Illumina Hiseq2500 platform at the LC-BIO (Hangzhou, China) following the vendor's recommended

protocol. CleaveLand 3.0 (Addo-Quaye *et al.*, 2009) was used for analysing sequencing data. The raw data of degradome sequencing have been submitted to the NCBI GEO datasets under the accession number SRP127561.

Investigation of the target mRNAs of miRNAs by qRT-PCR

Total RNA was extracted from the leaves with TRI Reagent (Sigma) according to the manufacturer's instructions, and the samples were treated with DNase I enzyme (Promega). Reverse transcription was performed using M-MLV Reverse Transcriptase and Oligo(dT) 15 primer (Promega) according to the manufacturer's instructions. The expression level of the target genes was determined by real-time RT-PCR using recently planned primers (Table S1A). The reactions were run on a CFX96 Real-Time PCR instrument (Bio-Rad) and the relative fold change (FC) values were calculated according to (Boldizsár *et al.*, 2016).

Computational prediction and analysis of miRNA targets

The targets of the H_2O_2-induced miRNA collection were predicted with the psRNA Target tool (http://plantgrn.noble.org/psRNATarget/). To confirm the degradome sequencing results and to determine additional target genes the EnsemblPlants 31 release of wheat nucleotide sequences were used as query cDNA library and the default scoring schema were used with the following parameters: (1) # of top targets=200; (2) Penalty for G:U pair=0.5; (3) Extra weight in seed region=1.5; (4) # of mismatch allowed in seed region=2; (5) Allow bulge (gap) on target=enabled; (6) Penalty for opening gap=2; (7) Calculate target accessibility=disabled; (8) Translation inhibition rate=10-11 NT; (9) Expectation=5; (10) Penalty for other mismatches=1; (11) Seed region=2-13 NT; (12) HSP size=19; (13) Penalty for extending gap=0.5.

MapMan (Thimm *et al.*, 2004) (https://mapman.gabipd.org/) and KEGG (http://www.genome.jp/kegg/) pathway database were used for annotation of the miRNA target genes. In addition, custom Blastx search was performed against the UniProt protein database (http://www.uniprot.org/downloads) and the predicted miRNA targets using a Geneious software version 9.8.1 (Biomatters, New Zealand; http://www.geneious.com).

The functional annotations were extended with gene expression data using an oligonucleotide-based microarray (E-MTAB-6627:

https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6627/). Preparation of Cy5- and Cy3-labelled cDNA using RNA isolated from the control and H_2O_2-treated samples, respectively, and microarray hybridisation to a stress-specific 15k wheat oligonucleotide microarray (Szűcs *et al.*, 2010) were performed as described (Szécsényi *et al.*, 2013). An Agilent scanner (Agilent, Santa Clara, CA, USA) was employed for microarray scanning and data collection as described previously (Kalapos *et al.*, 2016). The validation of microarray was done by qRT-PCR as described for the target mRNAs of miRNAs and the primers are listed in Table S1B.

The pathway map of the miRNAs and their target genes were build and visualized using the yEd graph editor version 3.18.0.2 (yWorks, Germany; https://www.yworks.com/products/yed).

Statistical analysis

Biochemical data from three independent experiments involving three biological repetitions each were evaluated, and standard deviations are indicated in the figures. The statistical analysis was prepared using one-way ANOVA and a least significant difference test or a Dunnett T3 non-parametic test (if any condition had not been-fulfilled) (SPSS program). The homogeneity of variances was tested by Levene's test. The relationships between the various parameters were checked by correlation analysis (Excel program).

Results

Growth parameters

Treatment with H_2O_2 resulted in a transient wilting and rolling of the leaves after 1 h (Fig. 1). After 2 h treatment the leaves of the treated plants recovered and were similar to the untreated ones. However, the fresh and dry weight and the dry weight/ fresh weight ratio of the shoots and roots were not affected by the H_2O_2 treatment (Fig. S1).

Effect of H_2O_2 treatment on the non-enzymatic components of the AsA-GSH cycle

Despite the visible effect of H_2O_2 on the plants after 1 h, there was no difference in H_2O_2 content between control and treated plants (Fig. 2). Subsequently a slight, gradual increase was observed in the H_2O_2 content during the one-day long experiment in the leaves of both untreated and H_2O_2-treated plants resulting in significant differences in certain sampling points compared to the starting value.

The amount of DHA and the DHA/AsA ratio greatly decreased in the control and treated leaves (to 50% or lower values compared to the starting value) while the amount of ASA and the redox potential of the DHA/AsA redox couple did not change during the experiment except for AsA in the control leaves after 24 h (Figs. 3A and 3B).

The concentration of GSH greatly increased after 3 h and remained nearly at this level until the 6-hour sampling in control plants and decreased after 6 h in the leaves of the treated seedlings compared to the starting value, which resulted in great differences between them (Fig. 4A). After the H_2O_2 treatment, its minimum values were detected in the dark. The GSSG concentration significantly increased at all sampling points under control conditions and after 3 h H_2O_2 treatment compared to the starting value. The GSSG/GSH ratio was greater both in the control and treated seedlings than its initial value throughout the experiment. The half-cell reduction potential of the GSSG/2GSH pair had a great increase after 6 h H_2O_2 treatment and further on (Fig. 4B). In contrast to GSH, the amount and redox state of its precursor, cysteine was not or only slightly affected by H_2O_2 (Fig. S2). The cysteine concentration was greater only after 24 h in the control seedlings. The cystine content increased in untreated leaves and remained unchanged in the treated ones except for the 3 h sampling compared to the starting value. The cystine/cysteine ratio varied between 17.2 and 25.5% and the half-cell reduction potential of this redox couple did not change during the whole experiment in both groups of plants.

Effect of H_2O_2 on the activity of antioxidant enzymes

The activities of the antioxidant enzymes are given on a protein basis. Concentration of protein did not change during the one-day experiment and was not affected by the H_2O_2 treatment (Fig. S3). The activity of CAT involved in the degradation of H_2O_2 increased both in the control and treated seedlings during the experiment, and it returned to the starting value after 24 h in the control seedlings, but remained high in the treated ones (Fig. 5A). However, the activity of APX, removing H_2O_2 in the AsA-GSH cycle increased even after 1 h treatment and was significantly greater compared to the starting value throughout the experiment while

such difference was observed for the control plants only after 3 and 12 h (Fig. 5B). The activity of GR, which is also an enzymatic component of the AsA-GSH cycle like APX, increased by 50% in both group of plants during the experiment and, after 24 h, it returned to the initial value in the control seedlings but not in the treated ones (Fig. 5C). The activity of GST, involved in the detoxification of xenobiotics and peroxides through catalysing their conjugation with GSH, exhibited a similar tendency of changes as GR (Fig. 5D). However, the increase in the activity in most sampling points (3, 6, 9, 12 h) compared to the starting value and the difference between the control and treated seedlings after 24 h was greater, about 2-fold.

Determination of the H_2O_2-responsive miRNAs

Small RNA sequencing was performed with samples collected after 0, 3 and 6 h treatment repeated in 2 parallels, which means 6 sequencing in total. After removing the low-quality and contaminant (length less than 18 nt or more than 30 nt reads) reads, 63.4 million reads were obtained in total and among them 11.6 million were mapped as unique ones (Table S2). The length distribution of the various small RNAs was similar in the different samples (Fig. S4). The proportion of redundant reads was the highest (25%) for the 21 and 24 nt long sequences and the proportion of unique sequences was the highest (60%) for the 24 nt long sequences.

We determined the differential expression of known wheat miRNAs (Sun *et al.*, 2014) between H_2O_2-treated and control seedlings. We found that a total of 70 miRNAs had a minimum 1.5-fold difference between control and H_2O_2-treated seedlings and they formed 7 groups on the basis of the time-course of changes in their expression after 3 and 6 h treatment compared to the starting value (Fig. 6). A transient increase was detected in the expression of 6 miRNAs after 3 h (group I), while the transcript level of 18 miRNAs (group V) remained also high after 6 h. The expression of 7 miRNAs (group II) decreased transitionally after 3 h and that of 6 miRNAs (group VII) was low after 6 h, too. Among the miRNAs the amount of which exhibited no or slight changes during the first 3 hours of the treatment, the expression of 21 (group III) and 10 (group IV) increased and decreased after 6 h, respectively. Interestingly, the transcript level of 2 miRNAs (group VI) was lower after 3 h and higher after 6 h compared to the value detected before the H_2O_2 treatment.

The transcript levels of 8 H_2O_2-responsive miRNAs with a minimum 2-fold change in their expression after 3 and/or 6 h compared to the starting value based on the sequencing results (Table S3) were further checked in a time-course experiment with 7 sampling points by qRT-PCR (Fig. 7). The tendency of H_2O_2-induced alterations obtained by the next generation sequencing after 3 and 6 h treatment was confirmed by these measurements (for tae-miR3106a and tae-miR3523a only in comparison with the starting value as done by the sequencing) and tae-miR2007a, tae-miR3147a and tae-miR3523a were already induced after 1h H_2O_2 treatment. Without treatment, the expression of the selected miRNAs exhibited a daily pattern: in several cases with higher levels during the light period and lower ones during the night (Figs. 7A, 7C, 7F and 7H). This pattern was modified by the H_2O_2 treatment. The miRNAs' levels were at least 2-fold greater after 24 h H_2O_2 treatment compared to the control values except for miR3106a having a 50% decrease in its expression. For tae-miR2007a, tae-miR818h and tae-miR3074a this difference was 9-fold or greater. The miRNA levels were minimum 4-fold greater for miR3106a after 3 h and 12 h and for miR3074a after 1 h in control seedlings compared to the treated ones in the same sampling point.

Identification of the target genes of the H_2O_2-responsive miRNAs

In order to determine those target genes of the H_2O_2-responsive miRNAs whose products are cleaved in our experimental system, RNAs obtained from samples taken after 0h, 3h and 6h H_2O_2 treatment were mixed equally for degradome sequencing. In this analysis 13.5 million raw reads were obtained and after the data analysis 160,466 covered cDNA sequences could be identified (Table S3). Based on the degradome sequencing, 86 unique target sequences of 28 H_2O_2-responsive miRNAs could be identified. For 29 target genes, the H_2O_2-responsiveness was also shown by microarray analysis (validated by qRT-PCR, r^2: 0.68, Fig. S5) and these genes are related to transcription, redox regulation, protein phosphorylation and degradation (Table S3). Two targets of tae-miR3493b, the genes encoding peroxidase 52 and a thioredoxin-like protein, and one target of tae-miR3513a encoding thioredoxin H8 are components of the antioxidant system. Both miRNAs were induced by H_2O_2 (Fig. 6, group III) and the expression of their targets were repressed as shown by microarray analysis (Table S3).

The time-course of changes in the expression of target genes of those 8 H_2O_2-responsive miRNAs shown in Fig. 7 was also investigated. Similarly, to the corresponding

miRNAs, the expression of their targets genes also exhibited a daily rhythm in the untreated seedlings, which was altered by H_2O_2. For several targets the direction of changes was opposite in the control and treated plants, which resulted in great differences between the transcript levels in certain sampling points (Figs. 8A, 8C, 8D, 8H). The expressions were influenced by the treatment already in the first 3 h and the difference between the control and treated seedlings was minimum 2-, but sometimes 10-fold at least in one sampling point for all target genes. Five of the targets were already induced after 1 or 3 h treatment (Figs. 8B, 8C, 8F, 8G and 8H), one only after 24 h (Fig. 8E) and two of them were repressed (Figs. 8A and 8D). A moderate negative correlation (r: -0.38 – -0.42) between the expression of the following miRNA and target pairs was found after H_2O_2 treatment: tae-miR2007A – ribulose-1.5-bisphosphate carboxylase activase, tae-miR3106 – beta-carotene isomerase, tae-miR3523a – glycerol-3-phosphate dehydrogenase. For 3 pairs a low negative correlation and for 2 pairs low positive correlation was observed.

Using bioinformatics tools 6808 unique target sequences (from them 86 were identified by degradome sequencing) of 70 H_2O_2-responsive miRNAs were found (Table S3). The following targets were related to the components of the AsA-GSH system or other antioxidants: tae-miR3369a – a phosphomannomutase involved in AsA biosynthesis, tae-miR3513a– monodehydroascorbate reductase, tae-miR3506b– peroxidase, tae-miR506b – GST, tae-miR3064a, tae-miR3510a, – thioredoxin. For 1647 targets the H_2O_2-responsiveness was also demonstrated by microarray study (Table S3).

Based on their targets, two types of KEGG-analysis of H_2O_2-responsive miRNAs were prepared using the database available for *Brachypodium*, a closely relative species to wheat (Table S5, Fig. S6). In the first one, the number of H_2O_2-responsive miRNAs was compared to the total number of the miRNAs in the individual categories in which the miRNAs were grouped based on their targets. The greatest number of such miRNAs was related to ‘plant-pathogen interaction (bdi04626)’, ‘protein processing in endoplasmatic reticulum (bdi04141)’, ‘carbon metabolism (bdi01200)’, ‘biosynthesis of amino acids (bdi01230)’, ‘purine metabolism (bdi00230)’, processes in ‘spliceosome (bdi03040)’ and ‘plant hormone signal transduction (bdi04075)’ (Fig. S6). In the second approach, the number of H_2O_2-responsive target genes was analysed. The two analyses gave different results since one miRNA may have several target genes and one target gene may be controlled by several miRNAs.

While the abundance of the H_2O_2-responsive miRNA in relation to the whole miRNA set in wheat was maximum 10% in the various KEGG-pathway categories (first evaluation approach), this ratio was at least 30% for 45% of target genes (second approach, Fig. S6). Taking into account the 7 greatest groups of categories, the same ones were selected by both approaches except for the 'purine metabolism (bdi00230)' by grouping of miRNAs and 'ribosome-related processes (bdi03010)' by grouping of targets. The enrichment of targets of H_2O_2-responsive miRNAs in the categories related to AsA and GSH metabolism, peroxisome, proteasome varied between 20-35%.

The possible interactions of the identified 70 H_2O_2-responsive miRNAs with their 6808 targets were also analysed (Fig. S7). This network with 9620 connections shows that most miRNAs have several targets, and most of the targets are controlled only by one miRNAs. However, several of them are regulated by two or more miRNAs therefore the miRNAs and their targets form a complex network.

In the central part of this network are located tae-miR818b, tae-miR818c, tae-miR818m, tae-miR818k, tae-miR818h, tae-miR3369a, tae-miR3523a and tae-miR3506b (Fig. 9) having 233 targets with 1437 connections. In this part of the network tae-miR3369a and members of the tae-miR818 family control the expression of many genes at translational level, while tae-miR3506b and tae-miR3523a does so by cleavage of their target mRNAs (Table S3). Most targets of these 8 miRNAs are involved in transcriptional regulation, protein phosphorylation and degradation (Table S6A). Based on KEGG categories, they were mainly (3-8 targets/ category) related to the processes in spliceosome, biosynthesis of amino acids, protein processing in ER, carbon, purine, starch and sucrose metabolism (Table S6B). Using a microarray analysis, the level and direction of the expression changes of several (1647) H_2O_2-responsive target genes were also determined (Table S3). From the targets of tae-miR3369a, 25 were present on the array and among them the expression of 12 and 6 genes increased and decreased, respectively. It is worth mention that for miR3493b, 16 of the 35 targets and for miR156a, 4 of the 17 targets were also identified by degradome sequencing.

Discussion

Effect of H_2O_2 on the redox environment in the leaves

Induction of oxidative stress is a common consequence of abiotic stresses. This effect was successfully simulated by H_2O_2 treatment previously in wheat and maize as shown by the modification of the redox environment (Kellős *et al.*, 2008; Gulyás *et al.*, 2014). Although the effect of H_2O_2 on the miRNA profile was investigated in rice and *Brachypodium*, the possible involvement of the AsA-GSH cycle and other antioxidants in the control of miRNA levels was not studied in these experiments (Li *et al.*, 2011; Lv *et al.*, 2016). In the present experimental system, H_2O_2 treatment efficiently modified the GSH-dependent redox environment and activated the related protective mechanisms in the leaves of wheat seedlings therefor the growth was not affected as shown by fresh and dry weight data. The H_2O_2-induced transient wilting and rolling of the leaves, as a part of the protection, may be due to a rapid loss of water content by the opening of stomata induced by transient local changes in H_2O_2 concentration since H_2O_2 signaling is involved in the control of stomatal movement (Hua *et al.*, 2012). The alterations in the GSH-dependent redox environment are indicated by the decrease in GSH content and increase in $E_{GSSG/2GSH}$ value compared to the untreated control plants. After a 3 h treatment when the first sampling for miRNA sequencing was prepared, the GSH content was by 30% lower in the treated seedlings than in the controls. In contrast to GSH, the AsA concentration and $E_{DHA/AsA}$ value did not change which can be explained by the 3 times greater size of the AsA pool compared to the GSH pool. APX, having an increased activity throughout the H_2O_2 treatment could successfully decompose the unnecessary H_2O_2 in plants deriving from its addition to the nutrient solution. Thus, no change in the endogenous H_2O_2 concentration was detected in leaf tissue extracts which does not exclude the local changes in its level in specific cells (stomata) and organelles (chloroplasts). However, during the removal of H_2O_2 in the AsA-GSH cycle, the amount and redox state of GSH also changed in leaf extracts because of its oxidation by GR having increased activity after 24 h treatment. In addition, the larger use of GSH by GST after 24 h for the detoxification of peroxides in the H_2O_2-treated plants will also reduce the GSH concentration. Besides, APX, CAT also could greatly contribute to the degradation of H_2O_2 since its activity was greater by 60% after 24 h in the treated seedlings compared to the control ones. The correlations between the expression levels of miRNAs, their target mRNAs and antioxidants (GSH, APX, GST, CAT) in the treated seedlings show the closeness and direction of their relationships (Table S7), The control of miRNAs by GSH was corroborated for miR395 in S-deprived *Arabidopsis*, since the expression of this miRNA was modified after addition of exogenous GSH or in GSH-deficient mutants (Jagadeeswaran *et al.*, 2014). In addition, tae-miR395 was H_2O_2-responsive in wheat (present study) and in *Brachypodium* (Lv *et al.*, 2016).

These results indicate that the effect of H_2O_2 on miRNAs is mediated by certain components of the AsA-GSH cycle, which has a central role in the redox regulation (Foyer and Noctor, 2011).

Effect of H_2O_2 on miRNA profiles

The H_2O_2-induced modification of the GSH-dependent redox environment had a great effect on the miRNA profile since a minimum 1.5-fold change was shown by the sequencing results in the expression of 70 miRNAs. Far more miRNAs were upregulated (44) than downregulated (22). Furthermore, some of them (4) was inhibited first (after 3 h) and induced afterwards (after the subsequent 3 h) in wheat. Similarly to wheat, a large number of miRNAs (61) was affected by H_2O_2 in *Brachypodium* (Lv *et al.*, 2016) while only 7 in rice (Li *et al.*, 2011). The low number of H_2O_2-responsive miRNAs in rice is surprising, since the developmental stage of seedlings (2-3 leaves), the applied concentration of H_2O_2 (10-20 mM) and the duration of treatment (2-6 h) were similar in all 3 species. These results indicate the greater sensitivity of the miRNA-related regulatory system to oxidative stress in the phylogenetically nearer wheat and *Brachypodium* compared to the more distant rice.

The ratio of H_2O_2-responsive miRNAs compared to their total number was very similar (mainly 1:10) within the various KEGG-categories in wheat. Among the categories with the greatest number of H_2O_2-responsive miRNAs were carbon metabolism, peroxisome-associated reactions including the glyoxylate pathway. These processes are related to the chloroplasts and peroxisomes (Slesak *et al.*, 2007), the major organelles responsible for the production of H_2O_2 explaining the large number of H_2O_2-responsive miRNAs in the metabolic pathways occurring in these organelles. Besides the H_2O_2 formation, peroxisome and chloroplast are also connected to the redox system through the formation of glycine and γ-glutamylcysteine, respectively, since these compounds are precursors of GSH. Two other main KEGG-categories with high number of H_2O_2-responsive miRNAs were the 'protein processing in the endoplasmatic reticulum (bdi04141)' and the 'proteasome-related degradation of proteins (bdi03020)'. The endoplasmatic reticulum is the main source of H_2O_2 in the cytosol (Slesak *et al.*, 2007), and proteasomes are protein complexes where H_2O_2-mediated S-glutathionylation of proteins takes place if the cellular redox state shifts (Jung *et*

al., 2014). In this process the H_2O_2-dependent alteration in the ratio of GSH/GSSG may affect the metabolism of proteins by the involvement of miRNAs.

Although several H_2O_2-responsive miRNAs were found in wheat (total H_2O_2-responsive: 70) and *Brachypodium* (total H_2O_2-responsive: 61) (Lv *et al.*, 2016), only two of them, tae-miR160b and tae-miR395a were common between the two plant species (Table S8). Tae-miR395a is involved in sulphate reduction and therefore indirectly in the GSH formation through cysteine; tae-miR160b has auxin-related function. Thus, the basic regulatory processes such as hormonal and redox regulations are conserved between these two species. While no overlap of H_2O_2-responsive miRNAs was observed between wheat and rice, miR169d, miR827-3p, miR397a and miR408-5p were affected by H_2O_2 both in *Brachypodium* and rice (total H_2O_2-responsive: 7) (Li *et al.*, 2011; Lv *et al.*, 2016). They regulate the genes encoding HAP2-like transcription factor, SPX-domain protein (regulation of phosphate homeostasis), laccase (lignin biosynthesis) and a monosaccharide transport protein, respectively. The limited overlap in H_2O_2-related miRNAs between the three species indicates the specificity of the miRNAs in the various organisms during the response to environmental changes.

Similarly, to H_2O_2 in wheat, ozone-induced oxidative stress also affected the members of the miR156 family (controlling flowering, yield and leaf initiation) in *Arabidopsis* (Table S8) (Iyer *et al.*, 2012). However, the members of the other 21 ozone-responsive miRNAs family were not influenced by H_2O_2 in wheat. Between rice and *Arabidopsis*, also only one common oxidative stress-responsive miRNA family, the miR169 (target: HAP2-like transcription factor), exists, which is involved in the stress-response (Li *et al.*, 2011; Iyer *et al.*, 2012). Members of miR169 family were also induced by H_2O_2 in *Brachypodium* (Lv *et al.*, 2016). In addition, miR160a (target: auxin response factor 22) and miR164 (target: phytoene dehydrogenase) were induced both in *Arabidopsis* and in *Brachypodium* by oxidative stress. These experiments indicate that different sets of miRNAs are involved in the response to various oxidants such as H_2O_2 and O_3. In addition, the response of the members of the same miRNA family to the various abiotic stresses in generally also differs in the different plant species. However, some similarities can also be found when comparing the miRNA set induced by oxidative and various abiotic stresses in *Arabidopsis* (Zhang, 2015; Barciszewska-Pacak *et al.*, 2015). Therefore, it is difficult to describe the involvement of the individual miRNAs in the stress response with a general model. The differences between the plant

501 species may be due to the complex regulatory networks of miRNAs in which the role of the
502 individual miRNAs varies between the species.

503 When checking the effect of H_2O_2 on miRNAs by qRT-PCR in a time course
504 experiment during 1 day, light-dependent daily changes were observed in their levels even in
505 the untreated seedlings, which observation corresponds with the light-responsiveness of
506 miRNAs demonstrated in *Brassica rapa* (Zhou *et al.*, 2016). A redox control of these light-
507 dependent daily alterations can be supposed since it was modified by H_2O_2 in wheat. The
508 tendency of changes in the expression of the miRNAs detected by sequencing after 3 and 6 h
509 treatment with H_2O_2 could be confirmed by qRT-PCR. After 9, 12 and 24 h exposure to H_2O_2,
510 the expression of those miRNAs also differed very often that were grouped together based on
511 the initial changes after 3 and 6 h H_2O_2 treatment in their transcription. Only some miRNAs
512 with an increase after 6 h H_2O_2 treatment (tae-miR3147a, tae-miR3074a) exhibited similar
513 time-course of expression changes after 9, 12 and 24 h of H_2O_2 addition. The observed large
514 increase in the expression of these 2 miRNAs during the first 6 h was probably due to the
515 additive effect of H_2O_2 and light, since the transcript levels became far lower in the dark even
516 after 1 h. In contrast, the amount of tae-mir3106a still remained high after 1 h in the dark and
517 it decreased only after 4 h in the treated plants. The effect of light on H_2O_2-responsive
518 miRNAs is further supported by the fact that the expression of 5 of them increased again in
519 the light after the transient decrease in the dark during the 1-day H_2O_2 treatment.

520

521 *Function of the selected miRNAs based on their target genes*

522 By bioinformatics tools we could identify 6808 possible targets for the 70 identified H_2O_2-
523 responsive miRNAs. However, by degradome sequencing only 86 targets of 28 of them could
524 be determined indicating that only this small set is actually affected by miRNAs after 0, 3 or 6
525 h in our experimental system. This large difference could derive from the possible temporal
526 and spatial shift in the expression changes in miRNAs and their targets and the existence of
527 complex regulatory network including opposite miRNA regulators of the same target
528 (Kawashima *et al.*, 2009; Liang *et al.*, 2014). Thus, a temporal shift in the expression was
529 found for tae-miR3106 and its target, beta-carotene isomerase. Among the target genes
530 determined by degradome sequencing several were related to transcriptional regulation
531 (targets of tae-miR156a, tae-miR3294b, tae-miR3369a, tae-miR3493aa), protein
532 phosphorylation (targets of tae-miR3319b, tae-miR3332a), protein degradation (targets of tae-

miR3369a, tae-miR818b, tae-miR2001a, tae-miR399b), and redox regulation (targets of tae-miR3493a, tae-miR3513a) and the core part of our regulatory network model was also associated with these processes. The H_2O_2 responsiveness of these target genes was also confirmed by microarray analysis in wheat. In addition, the KEGG analysis confirmed the enrichment of the target genes of H_2O_2-responsive miRNAs in the categories related to protein metabolism and redox processes. Although the degradation of few target mRNAs was only checked in *Brachypodium* and rice (Li *et al.*, 2011; Lv *et al.*, 2016), the effect of H_2O_2 was shown for several redox- and protein decomposition-related proteins in these species, too (Wan and Liu, 2008; Bian *et al.*, 2015). These results are in accordance with those ones obtained in wheat with degradome analysis because of the occurrence of common functional categories such as redox regulation and protein degradation.

The present results indicate a feed-back regulation between the redox system and the miRNAs since the H_2O_2-induced modification of GSH level and APX activity affected several miRNAs the target of which control the levels of redox compounds in wheat (Fig. 10). Thus, by degradome sequencing, three miRNAs (tae-miR3493b – target genes: peroxidase 52 and a thioredoxin-like protein; tae-miR3513a – target gene: thioredoxin H8) and by bioinformatics tools, 6 miRNAs were found. This hypothesis was also corroborated in the case of miR395 controlling the synthesis of the GSH precursor, cysteine. It also proved to be H_2O_2-responsive in *Brachypodium* (Lv *et al.*, 2016) and its expression depended on the availability of GSH in *Arabidopsis* (Jagadeeswaran *et al.*, 2014). Besides the indirect control of miRNAs through various antioxidants, H_2O_2 may also directly affect miRNAs as it was suggested for heavy metals (Min Yang and Chen, 2013). Instead of linear relationships, a network of mutual interactions can be supposed among H_2O_2, other redox compounds, miRNAs and metabolism which allows a continuous redox-dependent adjustment of miRNA levels and the related metabolic processes (Fig. 10).

Although the first 6 h were very important in the response of miRNAs and their targets to H_2O_2 in wheat, *Brachypodium* and rice (Li *et al.*, 2011; Lv *et al.*, 2016), the subsequent changes are also determinative for the reaction to stress as it was observed in wheat for 8 miRNAs and their targets during a whole day time-course experiment. Similarly, to the 8 miRNAs selected for qRT-PCR, their target genes also exhibited a light-dependent daily rhythm in untreated seedlings. This observation is not surprising since the light intensity changes during the day, which affects the possible formation of ROS in the photosynthetic electron transport chain and subsequently the redox-sensitive miRNAs and their targets will

be influenced. In our experimental approach we could detect these light-responsive miRNAs and their targets probably by modifying the amount of ROS after the addition of H_2O_2 to the nutrient solution of the plants.

Although, for certain miRNA-target pairs, we observed the expected negative correlation, it was not detected for each of them. The lack of such relationship can be explained by the function of miRNAs as mobile signalling elements during the H_2O_2-dependent regulation of gene expression for which a model was recently established in *Arabidopsis* (Liang *et al.*, 2014). Based on this model miRNA can act in another cell, tissue or organ on their target genes. Consistent with this hypothesis, in the case of miR395 and its target involved in the sulphur assimilation, a positive temporal but negative spatial correlation was found in *Arabidopsis* (Kawashima *et al.*, 2009). Besides the mentioned dynamic changes in miRNA levels, such alteration also occurs in ROS levels because of the compartmentalisation of ROS production and removal (Noctor *et al.*, 2018) that allows even a more complex redox-dependent regulation of miRNA targets.

A further possible explanation for the lack of the expected negative correlation between the level of the miRNAs and their targets is the existence of such regulatory networks in which one target genes is regulated by several miRNAs, and one miRNA controls several targets in wheat. In addition, a positive post-transcriptional regulation of gene expression is also possible as described recently for miR171b controlling arbuscular mycorrhizal symbiosis in *Medicago trunculata* (Couzigou *et al.*, 2017). Thus, in our network the result of the negative and positive regulatory effects could be a very fine regulation with small changes. In this network tae-miR33506b, tae-miR3523a, tae-miR3369a and members of the tae-miR818 family have central position with many target through which they are interconnected with other miRNAs. Similar regulatory network of H_2O_2-responsive miRNAs and their targets was also proposed in *Brachypodium* in which also a large number of target genes is controlled by one miRNA (Lv *et al.*, 2016). This system allows a co-ordinated regulation of a large set of genes. Probably only a small part of the many possible targets of a miRNA is regulated in a certain time point depending on the organ, developmental stage, environmental effects and regulatory interactions of various signalling pathways as indicated by the large difference in the number of the targets detected by degradome sequencing and predicted by bioinformatics tools in wheat All small RNA libraries prepared for this study were sequenced with Illumina Hiseq 2000.

Conclusions

H_2O_2 can modify the redox state in wheat through its effect on GSH, APX and other antioxidants which in turn influence the expression of miRNAs and their target genes and subsequently the metabolism. A network of interactions between the components of this model can be proposed. From the 70 H_2O_2-responsive miRNAs, for 28 miRNAs 86 target genes were identified and these genes are related to transcriptional regulation, protein phosphorylation, protein degradation and redox regulation as shown by degradome analysis. Based on a bioinformatics analysis, a regulatory network of 70 miRNAs with 6808 unique targets was created, which enables the fine adjustment a large set of redox-responsive genes.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Effect of H_2O_2 treatment on the fresh and dry weight and dry to fresh weight ratio of shoots and roots.

Fig. S2. Effect of H_2O_2 treatment on the size and redox state of the cysteine pool.

Fig. S3. Effect of H_2O_2 treatment on the protein content.

Fig. S4. Proportion of redundant and unique reads in miRNA transcriptome.

Fig. S5. Regression analysis of gene expression results obtained by microarray and qRT-PCR.

Fig. S6. KEGG-analysis of H_2O_2-responsive miRNAs and their targets.

Fig. S7. The whole network of the H_2O_2-responsive miRNAs and their targets determined by degradome sequencing and bioinformatics tools.

Table S1. Primers used for the qRT-PCR analysis of miRNAs target genes and validation of microarray data.

Table S2. Results of small RNA sequencing.

Table S3. H_2O_2-responsive miRNAs and their target genes

Table S4. Summary of degradome sequencing.

Table S5. KEGG categories of the targets of H_2O_2-responsive miRNAs.

626 **Table S6.** Correlations between the time-course of changes during 1-day H_2O_2 treatments in
627 the level of antioxidants, selected miRNAs and their target mRNAS investigated by qRT-
628 PCR.

629 **Table S7.** Genes and their regulator miRNAs in the core network (A) and distribution of core
630 network (Fig. 9) miRNAs and their target genes in different KEGG metabolic pathways (B).

631 **Table S8.** miRNAs responsive to oxidative stress in two or more species.

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Figure legends

Fig. 1. Effect of 1 h treatment with 10 mM H_2O_2 on the plants.

Fig. 2. Effect of H_2O_2 treatment on the endogenous H_2O_2 concentration. Values indicated with an asterisk are significantly different from that of detected at the starting time. Horizontal white and black bars indicate the light and dark periods, respectively.

Fig. 3. Effect of H_2O_2 treatment on the size and redox state of the ascorbate pool. A: Concentration of AsA and DHA and the percentage of DHA compared to ASA (the numbers above the columns). B: Half-cell reduction potential of the DHA/AsA couple. Values indicated with an asterisk are significantly different from that of detected at the starting time. Horizontal white and black bars indicate the light and dark periods, respectively. C: Control; T: Treated.

Fig. 4. Effect of H_2O_2 treatment on the size and redox state of the glutathione pool. A: Concentration of GSH and GSSG and the percentage of GSSG compared to GSH (the numbers above the columns). B: Half-cell reduction potential of the GSSG/2GSH couple. Values indicated with an asterisk are significantly different from that of detected at the starting time. Horizontal white and black bars indicate the light and dark periods, respectively. C: Control; T: Treated.

Fig. 5. Effect of H_2O_2 on the activity of antioxidant enzymes. A: catalase, B: ascorbate peroxidase, C: glutathione reductase and D: glutathione S-transferase. Values indicated with an asterisk are significantly different from that of detected at the starting time. Horizontal white and black bars indicate the light and dark periods, respectively.

Fig. 6. Expression of H_2O_2-responsive miRNAs determined by comparative transcriptome profiling. Expression changes of framed miRNAs were validated by qRT-PCR (Fig. 7).

Fig. 7. Time-course of the expression changes of miRNAs during 1-day H_2O_2 treatment. Horizontal white and black bars indicate the light and dark periods, respectively. Values indicated with an asterisk are significantly different from that of detected at the starting time.

Fig. 8. Expression patterns of miRNA target genes after 1-day H_2O_2 treatment. A: PMP – peroxisomal membrane protein (TC458412); B: RA - Ribulose-1,5-bisphosphate carboxylase activase (CK215494); C: UGE - UDP-glucuronate epimerase (CA697618); D: UCTH7 - Ubiquitin carboxyl-terminal hydrolase 7 (CA612693); E: β-CI - beta-carotene isomerase (Ta#S52543088); F: IAA6 - Auxin-responsive protein (Ta#S61781874); G: ACP - ADP, ATP carrier protein 1 (CA665835); H: GPDH: Glycerol-3-phosphate dehydrogenase (TC402657). Values indicated with an asterisk are significantly different from that of detected at the starting time. The correlation coefficients between the expression of miRNAs and their targets are given for the H_2O_2-treated samples. Horizontal white and black bars indicate the light and dark periods, respectively.

Fig. 9. Network of the H_2O_2-responsive miR818 family members and their target genes. The whole network of the H_2O_2-responsive miRNAs and their targets determined by degradome sequencing or bioinformatics tools is shown in the Suppl. Fig. S7. Squares: miRNAs; triangles: target genes identified by both degradome analysis and bioinformatics tools, circles: target genes identified by bioinformatics tools, green filling indicates decrease and red filling indicates increase in the target gene expression based on microarray data, grey filling indicates the lack of expression data. Continuous lines: cleavage of the target, dashed lines: translational inhibition. The colour of the lines indicates the strength of the interaction.

Fig. 10. Proposed regulatory network of H_2O_2, various components of the redox system, miRNAs, their target genes and metabolic processes.

Figures

Fig. 1. Effect of 1 h treatment with 10 mM H_2O_2 on the plants.

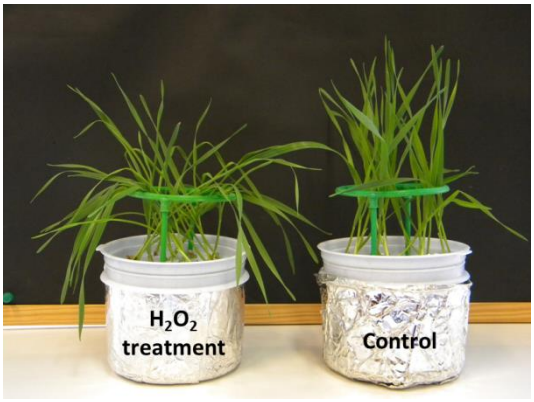


Fig. 2. Effect of H_2O_2 treatment on the endogenous H_2O_2 concentration.

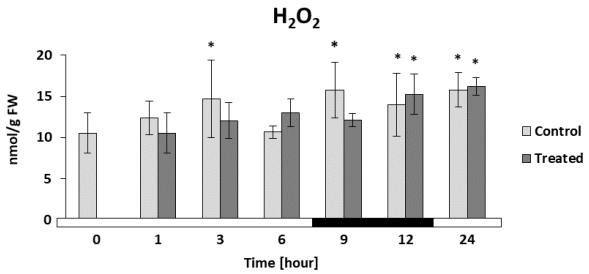


Fig. 3. Effect of H_2O_2 treatment on the size and redox state of the ascorbate pool.

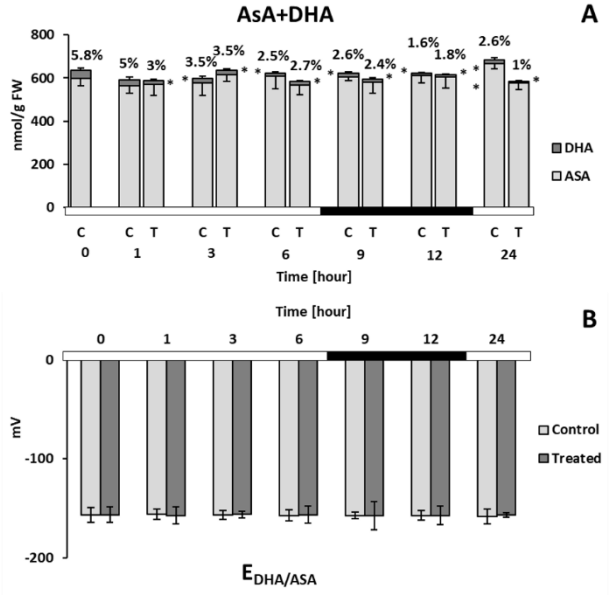


Fig. 4. Effect of H_2O_2 treatment on the size and redox state of the glutathione pool.

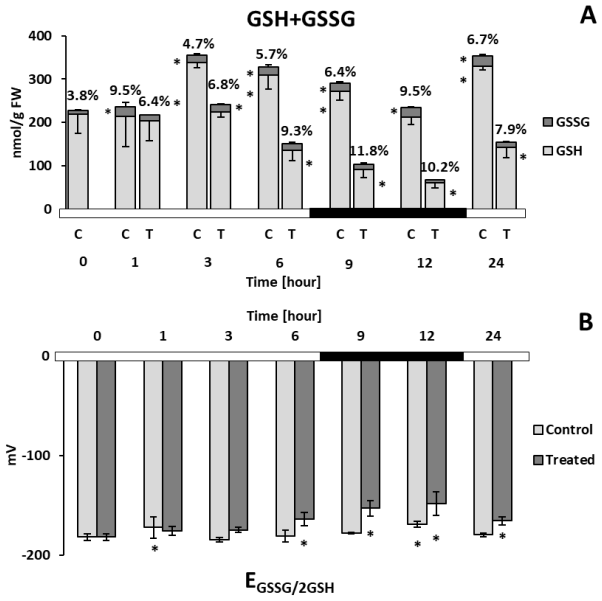
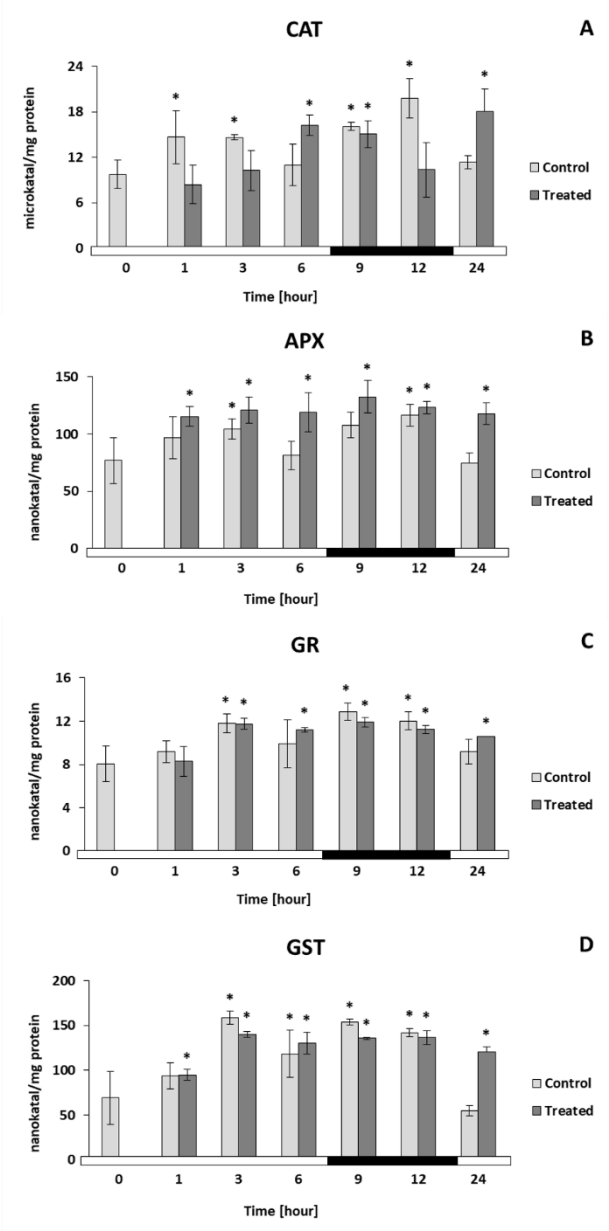


Fig. 5. Effect of H_2O_2 on the activity of antioxidant enzymes.



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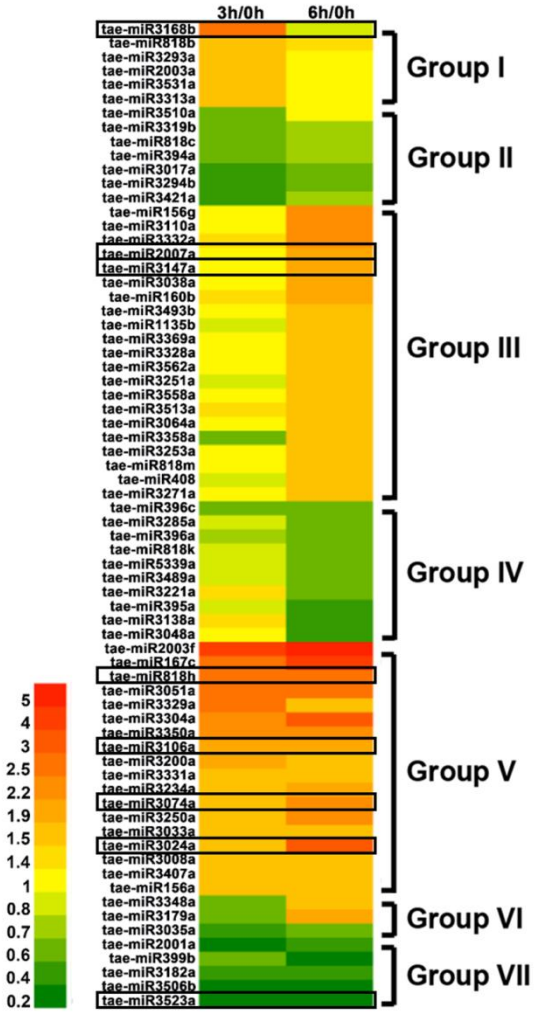

Fig. 7. Time-course of the expression changes of miRNAs during 1-day H_2O_2 treatment

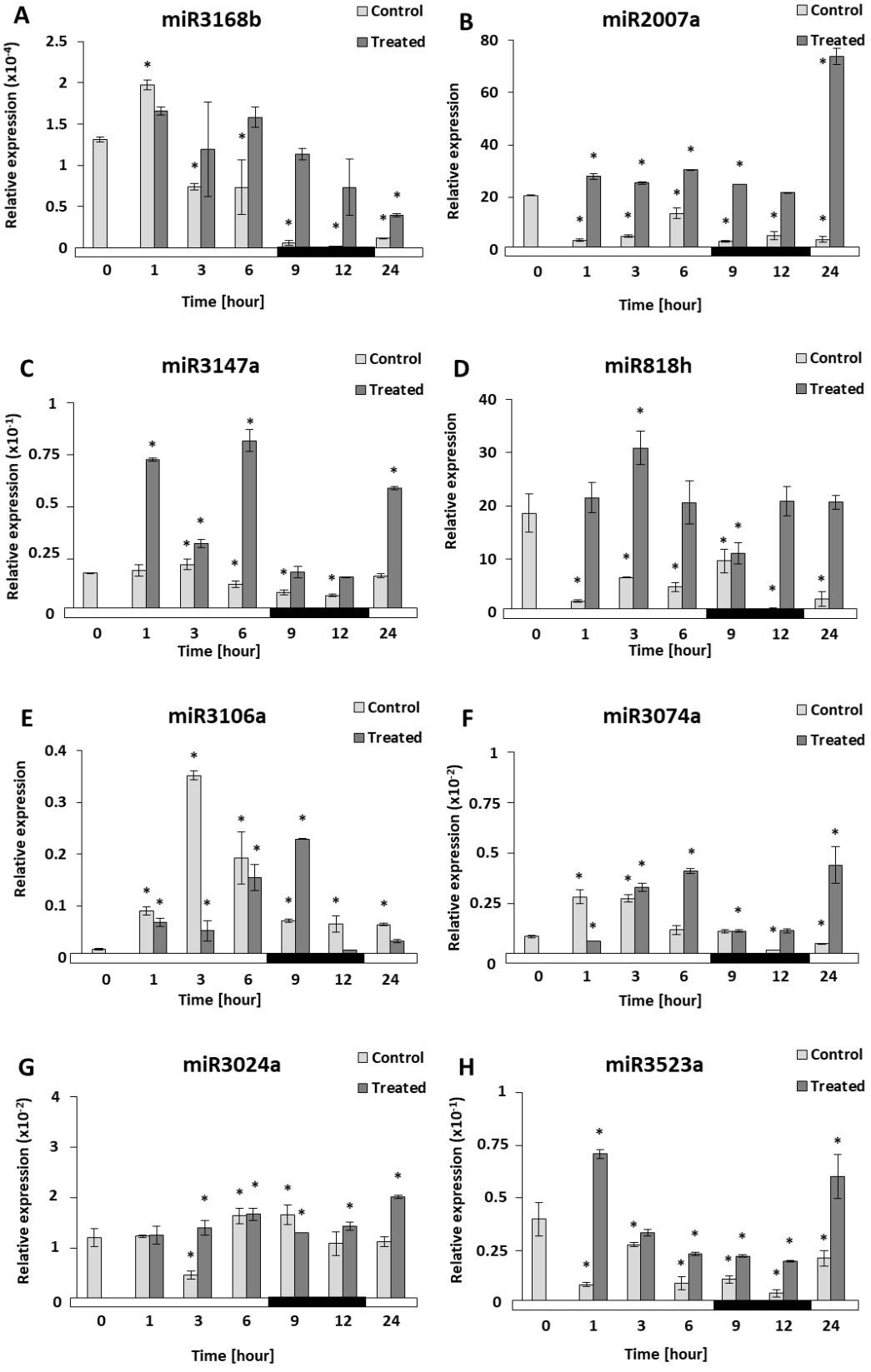

Fig. 8. Expression patterns of miRNA target genes after 1-day H_2O_2 treatment.

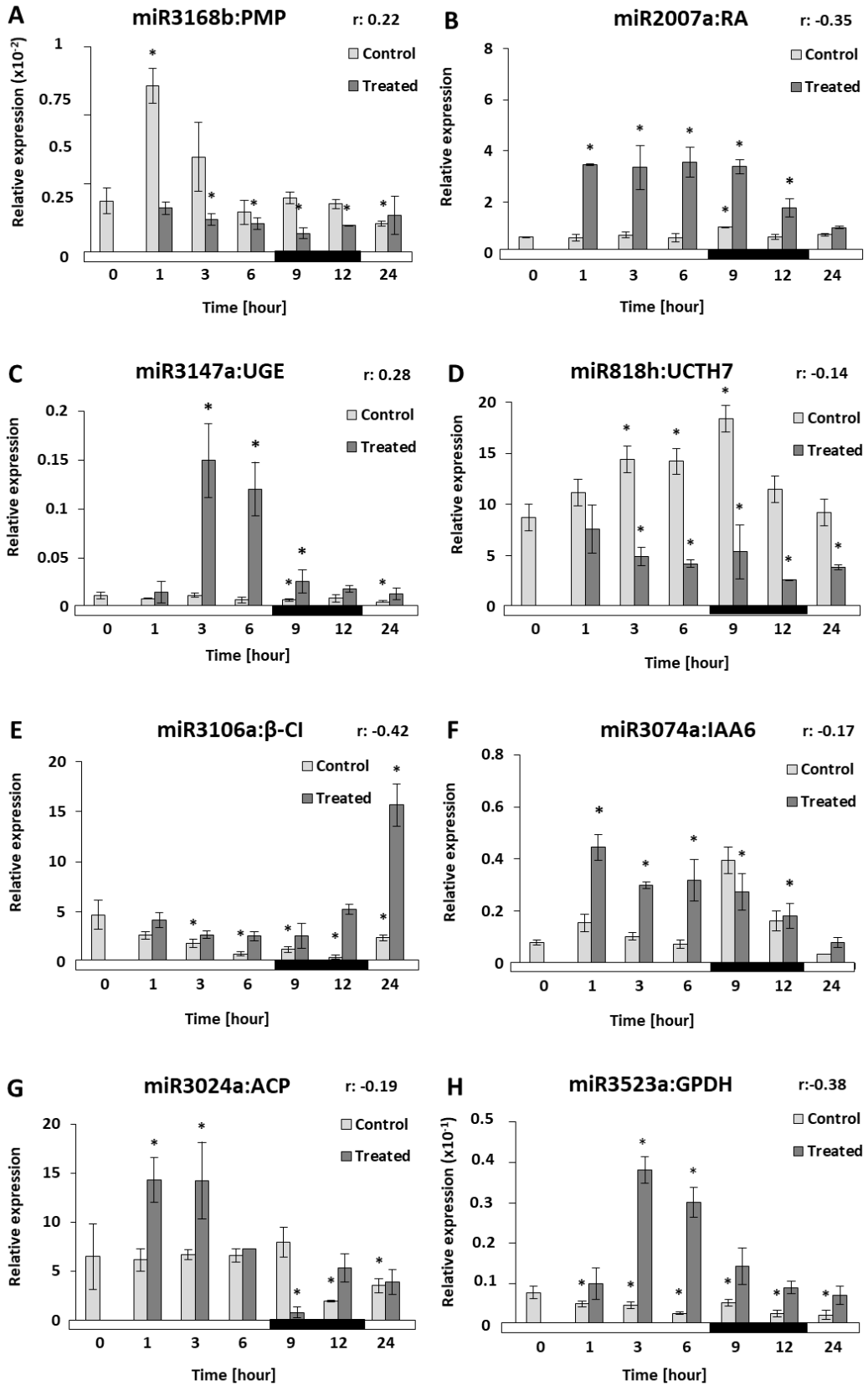

Fig. 9. Network of the H_2O_2-responsive miR818 family members and their target genes.

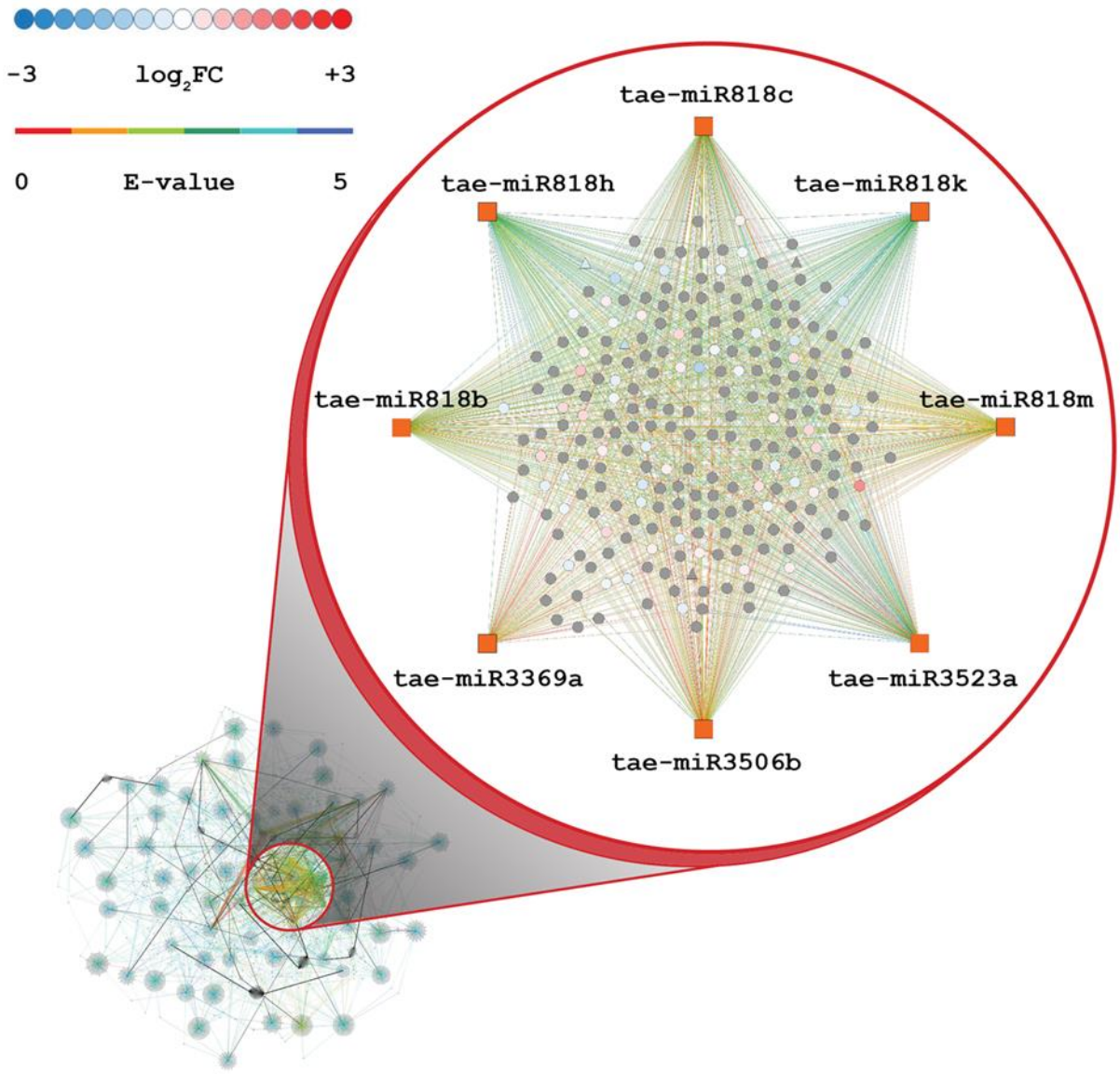

Fig.10. Proposed regulatory network of H_2O_2, redox system, miRNAs, their target genes and metabolic processes.