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Original Scientific Paper

TauL1, TauL2 and TauL3 gene-pools of *Aegilops tauschii* essentially differ in their genetic expression patterns

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ABSTRACT:
Aegilops tauschii is a wild diploid goat-grass which occupies a vast range in Central Eurasia and comprises three different gene-pools: TauL1, TauL2, and TauL3. Multivariate statistical analysis of the transcriptomes from the leaf tissue of 40 *A. tauschii* accessions, 18 of TauL1, 20 of TauL2, and 2 of TauL3, revealed that the gene-pools of *A. tauschii* distinctly and essentially differ in their genetic expression patterns. Statistically significant differential gene expression of 2349, 376, and 272 were observed between TauL1 and TauL2; TauL1 and TauL3; TauL2 and TauL3, respectively. These findings indicate substantial adaptive intraspecies divergence in *A. tauschii*.

Keywords:
adaptation, goat-grass, differentially expressed genes, intraspecies divergence, transcriptomes

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INTRODUCTION

Aegilops tauschii Coss. (syn. *Aegilops squarrosa* auct. non L.) is a wild diploid (genome DD, 2n = 14), primarily self-pollinating goat-grass whose D genome was incorporated into common wheat, *Triticum aestivum* L. (genome AABBDD, 2n = 42). As the most important wild relative of common wheat, *Aegilops tauschii* serves as a donor of agriculturally important genes for its improvement (Kimber & Feldman 1987; Kilian *et al.* 2011).

Multivariate statistical analysis of *A. tauschii* genetic variation revealed that the species comprises three distinctly different gene-pools (Dudnikov 1998, 2021; Pestsova *et al.* 2000; Mizuno *et al.* 2010), which were named TauL1, TauL2, and TauL3 (Matsuoka *et al.* 2013). According to intraspecies systematics based on *A. tauschii* spike morphology, TauL1 includes *Aegilops tauschii* Coss. subsp. *tauschii,* while TauL2 together with TauL3 consist of *A. tauschii* Coss. subsp. *strangulata* (Eig) Tzvelev (Eig 1929; Hammer 1980; Jaaska 1981; Dudnikov 2000, 2021; Pestsova *et al.* 2000; Zhou *et al.* 2021). Phylogenetic analysis revealed that early in its evolutionary history *A. tauschii* was represented by its subspecies *strangulata* with the TauL3 gene-pool being the most ancient, relict clade of this subspecies (Dudnikov 2012, 2017, 2019, 2021).

Aegilops tauschii inhabits patches of dwarf-shrub steppe-like formations, usually just below the edge of a forest belt, in hilly or mountainous regions in central Eurasia (Zhukovsky 1928; van Slageren 1994). Its habitats have not been disturbed by man since they are not suitable for any kind of agriculture. The species has a fragmented distribution, characterised by many small local populations (Dudnikov 1998). The TauL3

gene-pool was found only in Georgia and Dagestan (Russia). TauL2 occupies the area in Armenia, Georgia, Azerbaijan, Dagestan, Iran, and central Kopet-Dag in Turkmenistan. The TauL1 range is much wider, also including territories in Turkey, Uzbekistan, Kirgizstan, Tajikistan, Pakistan, India, and the Yellow River Basin (China) (Hammer 1980; Jaaska 1981; van Slageren 1994; Dudnikov 2000, 2021; Zhou *et al.* 2021). In the pre-Caspian area TauL2 is relatively more prevalent than TauL1, which tends to favour the continental part of the species range (Jaaska 1981; Dudnikov 2000, 2021).

The aim of the present study was to investigate the differences in gene expression between TauL1, TauL2, and TauL3.

MATERIALS AND METHODS

Plant materials. 40 *Aegilops tauschii* accessions representing all parts of the species range were used in the study: 18 of TauL1, 20 of TauL2, and 2 of TauL3 (Table 1). The sources of the plant material are as follows: (1) N.I. Vavilov All-Russian Institute of Plant Genetic Recourses (VIR), (accessions denoted by "k"); (2) Kyoto University ("KU"); (3) IPK Gatersleben ("AE"); Triticeae Research Institute, Sichuan Agricultural University ("As") and (4) the collection of Dudnikov (1998) ("t").

Molecular genetic methods. *Aegilops tauschii* plants belonging to different gene-pools were planted in random order and grown in a greenhouse under the same conditions at a temperature of 22 degrees Celsius, 80% humidity and round the clock illumination. RNA was extracted from the leaves of two-week old plants using a Qiagen RNeasy Plant Mini Kit. The quality of the RNA samples was evaluated using an Agilent Bioanalyzer 2100. All the samples showed a RIN value of 7.8 or higher. RNAseq library preparations were carried out with 1mkg of total RNA using a TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) according to the manufacturer's instructions for barcoded libraries with small modifications (4 min RNA fragmentation time and 12 PCR cycles were used). After normalisation the barcoded libraries were pooled and sequenced on a NextSeq550 sequencer 2 × 150 bp using a NextSeq 500/550 High Output v2.5 Kit 300 cycles (Illumina).

Data analysis. The obtained raw sets comprising about 20 billion 150 b.p. paired-end reads were analysed as follows. The adapters were removed by Cutadapt 3.5 (Martin 2011). Trimming and subsequent filtering was carried out using PRINSEQ 0.20.4 (Schmeieder & Edwards 2011). De novo transcriptome assembly was conducted using Trinity 2.13.2 (Grabherr *et al.* 2011; Haas *et al.* 2013). The transcriptomes were aligned to the reference genome using minimap 2 2.24 (Li 2018). (The Ensemble Plants reference genome Aet_v4.0 [Aegilops_tauschii.Aet_v4.0.dna.toplevel.fa.gz]). The FeatureCounts 2.0.1 programme (Liao *et al.* 2014) was used to create a read counts table. Differential expression analysis between the gene-pool pairs was carried out using the exact test devised by Robinson & Smith (2008) (Robinson *et al.* 2010) using Bioconductor edgeR 3.36.0.

A Venn diagram was created in Python (Supplementary Table 1).

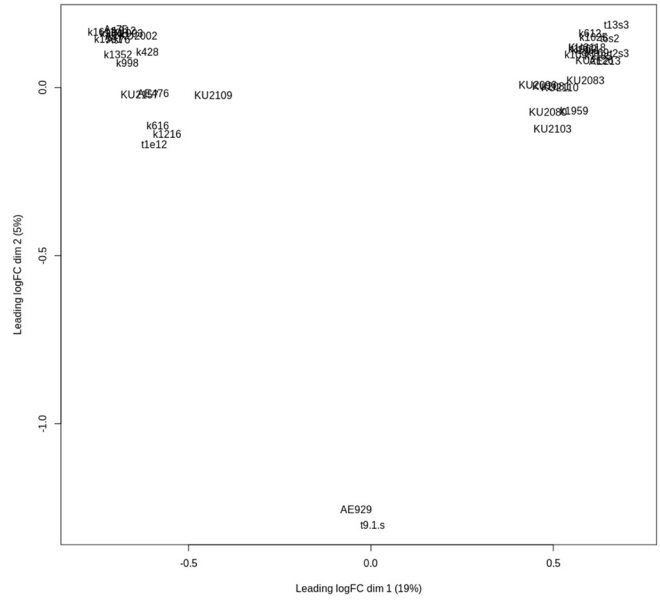

Fig. 1. A multi-dimensional scaling (MDS) plot created using the Bioconductor edgeR programme based on the levels of genetic expression in the transcriptomes of 40 *Aegilops tauschii* accessions.

RESULTS

Multidimensional scaling (MDS) of the *A. tauschii* transcriptome data revealed that genetic expression patterns distinctly and essentially differ in TauL1, TauL2, and TauL3. The three gene-pools were clearly separated as three distinct clusters on the plot of the first two MDS axes(Fig. 1).

Differential expression analysis identified statistically significant differential expression of 2349, 376, and 272 genes between TauL1 and TauL2; TauL1 and TauL3; TauL2 and TauL3, respectively. The results generated using edgeR (also including the lists of the top 10 genes, with the most divergent expression levels, for all three pairs of *A. tauschii* gene-pool comparisons) is given below. TauL1, TauL2, and TauL3 are referred to as group 1, 2, and 3, respectively. The tables show the gene identifier (gene ID), fold-change, which presents the difference between the groups (FC), counts per million, which measures the expression levels (CPM), and the p-value and false discovery rate correction (FDR) indicating the level of statistical significance of the difference in expression.

```
> et <- exactTest(y, pair=c("1","2"))
> topTags(et)
Comparison of groups:  2-1
                logFC   logCPM       PValue         FDR
AET2Gv20318000  6.652224 7.555387 3.361448e-98 1.332142e-93
AET2Gv20453400  6.295183 7.650268 2.962786e-97 5.870760e-93
AET2Gv20337000  6.700918 7.152962 6.034941e-66 7.972157e-62
AET3Gv20394800  6.181252 7.177832 1.884082e-65 1.866654e-61
AET4Gv20439100  6.559530 7.101891 3.171359e-62 2.295145e-58
AET6Gv20616300  5.048394 7.387833 3.474859e-62 2.295145e-58
AET6Gv20535200  6.706080 7.653218 8.914336e-61 5.046788e-57
AET5Gv20694200 -4.325267 7.210412 1.488758e-59 7.267484e-56
AET7Gv20801100  5.806613 7.555366 1.650451e-59 7.267484e-56
AET5Gv20753000 -5.340938 6.956176 2.607698e-59 1.033431e-55
> summary(decideTests(et))
       2-1
Down    815
NotSig 37281
Up     1534
> et <- exactTest(y, pair=c("1","3"))
> topTags(et)
Comparison of groups:  3-1
                logFC   logCPM       PValue         FDR
AET7Gv20862500 6.875125 6.722507 1.048853e-25 4.156606e-21
AET3Gv20490200 6.026753 6.965687 4.574621e-23 9.064611e-19
AET7Gv20801100 5.592331 7.555366 1.345795e-21 1.777796e-17
AET3Gv20595400 6.312094 6.659151 2.016763e-20 1.998108e-16
AET5Gv20077700 6.591431 5.450448 3.916759e-18 3.104423e-14
AET2Gv20452600 6.092753 6.222364 2.011985e-17 1.328916e-13
AET1Gv20809300 6.023437 7.284755 3.103192e-17 1.716640e-13
AET2Gv20453400 5.319733 7.650268 3.465334e-17 1.716640e-13
AET2Gv20600300 6.011523 6.574270 4.039178e-16 1.778585e-12
AET7Gv20504400 5.833943 6.917276 2.009247e-15 7.962648e-12
> summary(decideTests(et))
       3-1
Down     88
NotSig 39254
Up      288
> et <- exactTest(y, pair=c("2","3"))
> topTags(et)
Comparison of groups:  3-2
                logFC   logCPM       PValue         FDR
AET5Gv20753000  5.712572 6.956176 4.830108e-27 1.914172e-22
AET1Gv20251000  5.541347 5.490715 5.754879e-20 1.140329e-15
AET1Gv20398800  4.653255 7.026335 2.851191e-19 3.766423e-15
AET5Gv20077700  6.669707 5.450448 4.719898e-19 4.676239e-15
AET2Gv20318000 -7.240344 7.555387 6.817054e-16 5.403197e-12
AET2Gv20722200  5.708887 5.766931 5.813376e-14 3.291201e-10
AET3Gv21232800  5.708887 5.307494 5.813376e-14 3.291201e-10
AET2Gv21192800  5.222902 5.277120 7.451295e-13 3.691185e-09
AET3Gv20084300 -7.296364 7.691181 6.854304e-12 2.784804e-08
AET2Gv21074900  5.472885 5.230326 7.027010e-12 2.784804e-08
> summary(decideTests(et))
       3-2
Down    119
NotSig 39358
Up      153
```

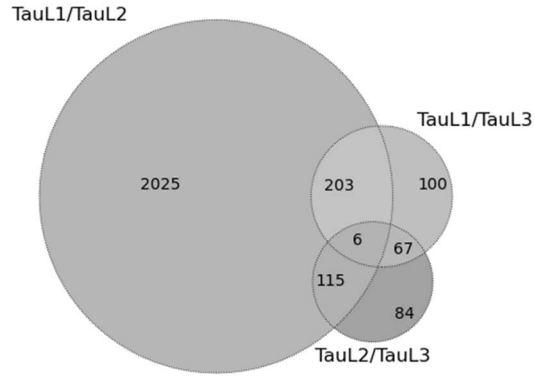

Fig. 2. Venn diagram. The number of genes identified as significantly differentially expressed in comparisons between TauL1, TauL2, and TauL3 gene-pools of *Aegilops tauschii*. 18 accessions of TauL1, 20 of TauL2, and 2 of TauL3 were used in the study.

Table 1. *Aegilops tauschii* accessions.

Accession	Subspecies*	Gene-pool	Country**	Locality***	Longitude	Latitude
					(Decimal)	
t 1e-12	t	TauL1	Russia	Dagestan, 3 km from Gedzhuh v. to Ersy v., 120 m	48.07	42.09
t 2s-3	s	TauL2		Dagestan, 4 km from Ersy to Gedzhuh, 200 m	48.04	42.07
t 6s-2	s	TauL2		Dagestan, vicinity of Rukel v., 380 m	48.24	41.98
t 9(1)s	s	TauL3		Dagestan, eastern slope of hill "336", 250 m	48.29	42.00
k 1025	s	TauL2		Dagestan, Derbent – Kuba road, 19 m	48.31	42.00
k 612	s	TauL2	Georgia	Gori d., vicinity of Rene v., 750 m	44.09	41.91
k 1216	t	TauL1		Aspindza d., 1000 m	43.25	41.58
AE 929	s	TauL3		Mccheta, Dzvari, S slope, 550 m	44.68	41.84
k 1185	s	TauL2	Armenia	Abovyan d., vicinity of Gehard, 1660 m	44.78	40.15
AE 476	t	TauL1		Erebuni	44.65	40.19
t 13s-3	s	TauL2	Azerbaijan	1.5 km from Maraza v. to Hilmilli v., 1000 m	48.88	40.64
k 109	s	TauL2		Astrahanbazar d., Novogolovnya v., 90 m	48.57	39.21
k 169	s	TauL2		Zakatali experiment station, 570 m	46.65	41.64
k 616	t	TauL1		SE of Shamhor	46.07	40.80
k 1961	s	TauL2	Iran (c)	Tage-Boston, 1400 m	47.07	34.28
KU 2118	s	TauL2		Khoy	44.96	38.55
KU 2157	t	TauL1		17.6 km SE from Karand to Shahabad, 1480 m	46.41	34.16
KU 2096	s	TauL2	Iran (wp)	51 km W of Babulsar (Babulsar - Chalus)	52.10	36.57
KU 2103	s	TauL2		13 km SSE of Resht (Chalus - Resht)	49.73	37.27
KU 2109	t	TauL1		Astara (Pahlavi - Astara)	48.86	38.41
KU 2110	s	TauL2		32 km SW of Astara (Astara - Ardabil)	48.55	38.40
KU 2126	s	TauL2		Techalousse (near Chalus)	51.42	36.64
k 1959	s	TauL2	Iran (ep)	Sari-Mazandaran, 10 m	53.04	36.57
k 1960	s	TauL2		Baijan, 1100 m	52.29	35.97
KU 2080	s	TauL2		Gharaghaj near Shahpesend (Gorgan - Hoshyeylak)	55.18	37.09
KU 2081	s	TauL2		NE of Koshyailagh (Gorgan - Koshyailagh)	55.45	36.94
KU 2083	s	TauL2		Koshyailagh	55.35	36.82
k 428	t	TauL1	Turkmenistan	Kara-Kala d., the road from Yari-Kala v. to Shermop, 320 m	56.32	38.38
k 1561	t	TauL1		Bolshoy Balhan mountain ridge	54.40	39.58
k 1903	t	TauL1		Kara-Kala d., Keshi-Yol mountain	56.50	38.45
AE 213	s	TauL2		Syunt-Hasardag mountain ridge, Mezetli mountains, 1400 m	56.58	38.54
k 964	t	TauL1	Afghanistan	Kabul – Kunduz road, S slope of Gindikush, before Salang pass, 1850 m	69.22	35.17
k 998	t	TauL1		Doshi – Bamian road, 940 m	68.44	35.57
KU 2002	t	TauL1	Pakistan	Suburbs of Quetta	67.00	30.21
k 912	t	TauL1	India	Kashmir, near Srinagar	74.86	34.07
k 1352	t	TauL1	Uzbekistan	Surhan-Darya region, Baysun, 1250 m	67.19	38.21
k 1611	t	TauL1	Kazakhstan	Alma-Ata region, Talgar d., 15 km from Novo-Alekseevka v., 800 m	77.22	43.41
As-75	t	TauL1	China	Xi'an, Shaanxi	108.94	34.34
As-76	t	TauL1		Sanmen Gorge, Henan	111.34	34.83
As-77	t	TauL1		Lushi, Henan	111.05	34.08

* "t" – ssp. *tauschii*; "s" – ssp. *strangulata*
** "(c)" – continental; "(wp)" – western precaspian; "(ep)" – eastern precaspian
*** "d." – district; "v" village

Table 2. Top differentially expressed gene (DEG) ontologies from Ensemble Plants.

TauL2 – TauL1			
	GO Term		
Gene	Biological process	Cellular component	Molecular function
AET2Gv20318000	GO 0006508 proteolysis		GO 0008234 cysteine-type peptidase activity
AET2Gv20453400			
AET2Gv20337000			GO 0008289 lipid binding
AET3Gv20394800			
AET4Gv20439100	GO 0006308DNA catabolic process GO 0006950 response to stress GO 0090305 nucleic acid phosphodiester bond hydrolysis GO 0090502 RNA phosphodiester bond hydrolysis endonucleolytic		GO 0000014 single-stranded DNA endodeoxyribonuclease activity GO 0003676 Nucleic acid binding GO 0004518 nuclease activity GO 0004519 endonuclease activity GO 0004521endoribonuclease activity GO 0016787 hydrolase activity GO 0016788hydrolase activity, acting on ester bonds GO 0046872 metal ion binding GO 0051879 Hsp90 protein binding
AET6Gv20616300			
AET6Gv20535200			
AET5Gv20694200			
AET7Gv20801100	GO 0051276 chromosome organization	GO 0005694 chromosome	GO 0005515 protein binding GO 0005524 ATP binding
AET5Gv20753000			
TauL3 – TauL1			
AET7Gv20862500	GO 0006486 protein glycosylation	GO 0016020 membrane	GO 0016758 hexosyltransferase activity
AET3Gv20490200			
AET7Gv20801100	GO 0051276 chromosome organization	GO 0005694 chromosome	GO 0005515 protein binding GO 0005524 ATP binding
AET3Gv20595400			
AET5Gv20077700	GO 0006520cellular amino acid metabolic process		GO 0016491 oxidoreductase activity
AET2Gv20452600			
AET1Gv20809300			
AET2Gv20453400			
AET2Gv20600300			
AET7Gv20504400			
TauL3 – TauL2			
AET5Gv20753000			
AET1Gv20251000	GO 0000737 DNA catabolic process, endonucleolytic GO 0006302 double-strand break repair		GO 0003677 DNA binding GO 0004518 nuclease activity GO 0008821 crossover junction endodeoxyribonuclease activity
AET1Gv20398800	GO 0006096 glycolytic process		GO 0003872 6-phosphofructokinase activity
AET5Gv20077700	GO 0006520 cellular amino acid metabolic process		GO 0016491 oxidoreductase activity
AET2Gv20318000	GO 0006508 proteolysis		GO 0008234 cysteine-type peptidase activity
AET2Gv20722200	GO 0030244 cellulose biosynthetic process	GO 0016020 membrane	GO 0016760 cellulose synthase (UDP-forming) activity
AET3Gv21232800	GO 0000723 telomere maintenance GO 0006281 DNA repair		GO 0003678 DNA helicase activity
AET2Gv21192800	GO 0000723 telomere maintenance GO 0006281 DNA repair		GO 0003678 DNA helicase activity
AET3Gv20084300	GO 0006508 proteolysis	GO 0005634 nucleus	GO 0008233 peptidase activity
AET2Gv21074900	GO 0000723 telomere maintenance GO 0006281 DNA repair		GO 0003678 DNA helicase activity

The three lists of differentially expressed genes (Supplementary Tables 2–4) were compared and the results are presented on a Venn diagram which displays the number of shared differentially expressed genes in the different comparisons (Fig. 2).

DISCUSSION

Multivariate statistical analysis of genetic variation using informative genetic markers, such as AFLP, SSR, DArT, SNPs, etc. (Pestsova *et al.* 2000; Saeidi *et al.* 2008; Takumi *et al.* 2008; Mizuno *et al.* 2010; Sohail *et al.* 2012; Matsuoka *et al.* 2013; Wang *et al.* 2013) in a representative set of *A. tauschii* accessions (genetic lines) reveals its distinct subdivision into the TauL1, TauL2, and TauL3 gene-pools. Even a set of allozyme markers is able to identify this subdivision of *A. tauschii* when analysing genetic frequencies in local populations (Dudnikov 1998).

Whatever set of genetic markers "scattered" through the *A. tauschii* genome is used in a study, it can be predicted in advance with 100% certainty that the analysis will show the three major gene-pools within the species, producing a similar pattern to that in Fig. 1. However, the results obtained in this study, shown in Fig. 1, are particularly surprising, since they are not based on the differences in the genetic sequences of the *A. tauschii* genetic lines studied, but on the levels of genetic expression. In this case it would be reasonable to expect a rather random spatial occurrence of *A. tauschii* accessions throughout the MDS plot. While there are some differences in spike morphology between *A. tauschii* subsp. *strangulata* (i. e. TauL2 + TauL3) and *A. tauschii* subsp. *tauschii* (TauL1), even experienced botanists are not always able to reliably distinguish these two subspecies. No morphological differences were noted between TauL2 and TauL3, and there is no evidence of any reproductive barrier within *A. tauschii*. Any accession can be crossed with another, exhibiting 100% fertility in subsequent generations (Dudnikov 2003). It is, therefore, surprising to observe such pronounced differences in the patterns of genetic expression between the three gene-pools within *A. tauschii*. It is known that *A. tauschii* subsp. *strangulata* (TauL2 + TauL3) and *A. tauschii* subsp. *tauschii* (TauL1) essentially differ ecologically (Dudnikov 2014). The study of *A. tauschii's* evolutionary history revealed that the intraspecies divergence was mostly adaptive (Dudnikov 2021). However, even taking this into account, it was hard to predict that the adaptive divergence would be so extensive as to result in the patterns of genetic expression shown in Fig. 1.

The aforementioned above genetic markers use non-coding DNA sequences, where variation is neutral. If a geographic barrier emerges and divides a population, after some time these genetic markers, working as "molecular clock", will inevitably present these two populations as different clades on a phylogenetic tree. And if the ecological conditions are the same on both sides of the barrier, the specimens from the different clades will not differ in terms of their adaptation peculiarities. On the contrary, when examining genes, different expressions of the same genes in the same ecological conditions in different specimens (of the same age and gender) of the same species reflect adaptive variability. Although such variability can be high among different species (i.e. independent genetic systems), within a single species in the same ecological conditions the same genes would be expected to express in the same way. The data obtained display that *A. tauschii* exhibits a very high level of adaptive genetic variation. Despite this it remains a single species.

Figure 1 shows that the differences in gene expression between TauL3 and TauL2, and between TauL3 and TauL1 were as pronounced as those between TauL1 and TauL2. At the same time, the number of statistically significant differentially expressed genes was considerably lower for the first two pairs of gene-pools than for the last one (272, 376, and 2349, respectively), a probable consequence of the very small sample size of TauL3 accessions involved in the study. The difference in gene expression between TauL3 and Taul2 was relatively lower than between TauL3 and TauL1 (272 and 376 differentially expressed genes (DEG), respectively). It is also evident that most of DEG from the TauL3/TauL1 pair (209 out of 376) were the same as in the TauL2/TauL1 pair, whereas the majority of DEG from the TauL3/TauL2 pair (151 out of 272) differed from those in the TauL2/TauL1 pair (Fig. 2). All this is in line with our knowledge that TauL2 and TauL3 belong to the same subspecies *A. tauschii* subsp. *strangulata* (Dudnikov 2021).

Ensemble Plants provides ontology information for many *A. tauschii* genes. This data for the top 10 mostly differentially expressed genes for each of the three pairwise comparisons is presented in Table 2. Unfortunately, for some genes the ontology information is missing. Future detailed analysis of the ontology of differentially expressed genes in TauL1, TauL2, and TauL3, and its correspondence with the ecological peculiarities of these gene-pools would be of great interest from both theoretical and applied perspectives. Clearly, any genetic variation with aids *A. tauschii* phylogenetic lineages in their adaptation in the wild could hold potential for *T. aestivum* breeding.

CONCLUSIONS

We found that the gene-pools of *A. tauschii* (TauL1, TauL2, TauL3) exhibit distinct differences in their genetic expression patterns, indicating a substantial level of intraspecies adaptive divergence. More than two thousand genes are differentially expressed in TauL1, TauL2, and TauL3, making these genes of particular interest for understanding the mechanisms of adaptive evolution in plant species. They are also very interesting from an applied point of view since *A. tauschii* is the most important wild species used as a source of genetic variation to improve common wheat, *T. aestivum*.

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REZIME

Botanica SERBICA

TauL1, TauL2 and TauL3 genski fondovi vrste *Aegilops tauschii* suštinski se razlikuju po obrascima genetske ekspresije

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Aegilops tauschii je diploidna vrsta koja zauzima veliki raspon u centralnoj Evroaziji i sastoji se od tri različita genofonda: TauL1, TauL2 i TauL3. Multivarijantna statistička analiza transkriptoma iz tkiva lista 40 *A. tauschii*, 18 od TauL1, 20 od TauL2 i 2 od TauL3, otkrili su da se njeni genski fondovi jasno i suštinski razlikuju po obrascima genetskog izražavanja. Ukazano je na statistički značajnu diferencijalnu ekspresiju 2349, 376 i 272 gena između TauL1 i TauL2; TauL1 i TauL3; TauL2 i TauL3 – respektivno. Dobijeni podaci su ukazali na veoma značajnu adaptivnu intraspecijsku divergenciju u *A. tauschii*.

Ključne reči: adaptacija, kozja trava, diferencijalno izraženi geni, intraspecijska divergencija, transkriptomi

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