Integrated physiological and transcriptomic analyses responses to altitude stress in oat (Avena sativa L.)

YU Jinqiu  
Northeast Agricultural University

Li Bing  
Northeast Agricultural University

Cui Guowen  
Northeast Agricultural University

Song Tingting  
Northeast Agricultural University

He Wenhua  
Qiqihar Grassland Station

Lian Lu  
China Agricultural University

He Jinglei  
Northeast Agricultural University

Kong Ling zelai  
Northeast Agricultural University

Chen Yajun  (chenyajun622@163.com)

Research article

Keywords: Avena sativa L., Altitude stress, Transcriptome sequence, Differentially expressed genes

DOI: https://doi.org/10.21203/rs.3.rs-37220/v1

License: ☒ This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License
Abstract

**Background** The oat is an annual gramineous forage grass with the remarkable ability to survive under various stressful environments. However, understanding the effects of high altitude stresses on oats is poor. Therefore, the physiological and the transcriptomic changes were analyzed at two sites with different altitudes, low (ca. 2,080 m) or high (ca. 2,918 m) respectively.

**Results** It was indicated that oats at high altitude showed higher levels of antioxidant enzyme activities and significant decreases in the non-photochemical quenching coefficient, chlorophyll content and stomatal density. Furthermore, oat yields were severely suppressed at the high altitude. RNA-seq results showed that 11,639 differentially expressed genes were detected at both the low and the high altitudes in which 5,203 up-regulated and 6,436 down-regulated. GO and KEGG annotations suggested that many differentially expressed genes be involved in pathways of cellular processes, metabolic processes and plant-pathogen interactions. Furthermore, 125 differentially expressed genes encoding TFs were involved in signal transductions by regulating the resistance genes in oats at high altitude. Some differentially expressed genes related to pigment metabolisms and photosyntheses were differentially expressed suggesting that these genes probably play responsive and regulatory roles in oats under the high altitude stress. Meanwhile, 273 differentially expressed genes related to hormone signaling pathways were significantly enriched in gibberellin, auxin, jasmonic acid and abscisic acid pathways indicating that these genes participated in the processes of oats responding to high altitude stresses.

**Conclusion** In summary, our study generated genome-wide transcript profile and may be useful for understanding the molecular mechanisms of Avena sativa L. in response to high altitude stresses. These new findings contribute to our deeper relevant researches on altitude stresses and further exploring new candidate genes for adapting plateau environment oat molecular breeding.

**Background**

Extreme environments provide natural laboratories for studies on the processes of speciation and adaptive evolution of organisms [1]. The Qinghai-Tibetan Plateau, the highest plateau in the world, plays an important role in climate changes in Asia and even the world [2]. In particular, the Qinghai-Tibetan Plateau environment is known for its harsh conditions, characterized by severe coldness, intensive ultraviolet radiations, hypoxia, poor soils and low CO2 pressure [3, 4]. Therefore, the survival in Qinghai-Tibetan Plateau is very challenging for most organisms. Nevertheless, many plant species can thrive in the cold and hypoxic conditions in high-alpine areas [5, 6]. Generally, in response to the bioclimatic conditions, plants can make corresponding molecular and physiological changes[7]. For example, the high-altitudinal gradient can restrict plant growth and reproduction via strong solar UV-B radiation, resulting in a reduction of photosynthetic rates by bleaching chlorophyll a (Chl a) and damaging the photosynthetic apparatus[8]. As such, some alpine plants are able to activate antioxidants such as APX, CAT, GR, proline and abscisic acid to confer plants with the tolerance to the alpine environments [9–11].
In recent years, transcriptome sequencing has been proven to be an effective and efficient method for determining adaptive evolutions and differential gene expressions in high-altitude plants, e.g. *Kobresia pygmaea, Potentilla saundersiana, Lamiophlomis rotate* and *Lobelia*. Some studies demonstrated that alpine plants had various morphological and physiological response strategies to adapt to high-elevation environments. For example, researchers had revealed that some genes were significantly involved in energy metabolism, hypoxia response under positive selection and rapid adaptation [12–14]. However, up to now, few transcriptome-based investigations have been devoted to the molecular mechanisms of high-altitude adaptation in oats.

Oats have not only high yield, rich nutrition and good palatability, but also the characteristics of cold tolerance, drought resistance and strong adaptability. Oats can germinate when the temperature is 3–4 °C and seedlings can resist the low temperature of -3 to -4 °C. Oats are the main forage species in the Qinghai-Tibet Plateau where they are high-quality forages for both winter and spring feeding and disaster-preserving livestock in local farming and pastoral areas. Planting oat grass is of great significance for solving short pasture supply in winter and spring grassland and developing herbivorous livestock and animal husbandry[15]. The oat has also been identified as an excellent species that can adapt to various environmental stresses including drought, salinity and pathogen attacks. Oats make morphological and physiological changes to adapt to environments under abiotic stress[16, 17]. However, the adaptation mechanisms of oats in high altitude environments have been rarely studied.

In this study, the first step was designed to characterize the impact of altitudes on oat physiology and agriculture. It was showed that high altitude influenced photosynthetic ability, antioxidant enzyme activity, stoma aperture and agricultural parameters of oats. The second step, RNA-seq was employed to study the changes at molecular levels between low and high altitudes. According to extensive data analyses, many DEGs and metabolic pathways were identified and characterized which were involved in adaption of oats to high altitude stresses.

**Results**

1. **High altitude environments induced physiological changes in oats**

MDA is an important marker for the lipid peroxidation due to overproduction of ROS in the cell. As shown in Fig. 1A, the MDA activities of the high altitude oats were higher than those of the low altitude. In order to further exploring the oxidative damage of oats, the activities of SOD were investigated. Under high altitude conditions, the SOD activities of the enzymes increased significantly compared to those at low altitude (Fig. 1B). In addition, the high altitude induced the accumulation of soluble sugars in oats (Fig. 1C). The results showed that oats could improve the ROS scavenging system, MDA homeostasis and osmotic regulation system which could decrease the jury caused by the high altitude environments.

Moreover, the chlorophyll content and the NPQ were determined to compare the photosynthesis changes of oats in two different altitudes. As shown in Fig. 1D, E, the chlorophyll content and NPQ at the high altitude decreased and were significantly lower than those at the low altitude. This result indicated that
there was a strong photosynthetic apparatus damage in high altitude conditions. Stomata control the carbon dioxide absorption and play a crucial role in photosynthesis. The density of stomata was measured. This result suggested that the stoma density had significantly decreased at high altitude (Fig. 1F, G).

2. The agronomic traits of Oats were remarkably affected by altitudes

Oats displayed significant differences from two altitudes in terms of plant height, stem/leaf ratio, crude fat and total hay yield. Under high altitude conditions, the plant height of oats was 104.7 cm which was significantly shorter than 126.9 cm at the low altitude (Fig. 2A). The stem/leaf ratio and crude fat at the low altitude were 1.18 and 2.7%, respectively which were significantly different from 1.05 and 1.7% at the high altitude, (Fig. 2B, C). On the other hand, compared with the low altitude, the high altitude exhibited remarkable decreases in hay yield (Fig. 2D). It was clear that oats altered their agronomic traits in two different regions. All these differences between the two altitudes could be the results of distinct modifications under atmospheric pressure, UV radiation, day length, temperature or the combination among all or some of these factors. Therefore, further research may be needed to illuminate the specific functions of these climatic factors in oats.

3. Transcriptome Profiling

To identify the transcriptomes and gene expression profiles of oats at low and high altitudes, six cDNA samples from oat leaves were prepared and sequenced using Illumina HiSeq 2000 platform. A total of 47–50 million raw reads were harvested from each cDNA library. After the low quality reads were removed, 39.49 Gb clean reads were obtained with an average of 6.66 Gb reads for each sample. The percentage of bases higher than Q30 in each sample was not less than 90.91% (Table 1).

| Sample | Total Raw Reads (Mb) | Total Clean Reads (Mb) | Total Clean Bases (Gb) | Clean Reads Q20 (%) | Clean Reads Q30 (%) | Clean Reads Ratio (%) |
|--------|---------------------|-----------------------|------------------------|---------------------|---------------------|-----------------------|
| Low1   | 50.62               | 44.36                 | 6.65                   | 96.63               | 91.03               | 87.64                 |
| Low 2  | 50.62               | 44.63                 | 6.69                   | 96.59               | 90.91               | 88.16                 |
| Low 3  | 50.61               | 44.48                 | 6.67                   | 96.41               | 90.99               | 87.88                 |
| High1  | 50.62               | 45.01                 | 6.75                   | 96.77               | 91.30               | 88.92                 |
| High2  | 47.92               | 42.68                 | 6.40                   | 96.86               | 91.51               | 89.06                 |
| High3  | 50.62               | 45.19                 | 6.78                   | 96.78               | 91.34               | 89.27                 |

To predict and analyze the functions of the unigenes, we carried out functional annotations by using BLAST against multiple databases such as Nr, Nt, KEGG, Swissprot, KOG, Interpro, and GO. A total of
11,639 unigenes were successfully matched up in the databases mentioned above. Among them, 11,507 (12.12%), 38,504 (40.55%), 12,044 (12.68%), 11,801 (12.43%), 12,154 (12.80%), 15,199 (16.00%), 49,383 (52.00%) unigenes were found in Nr, Nt, KEGG, Swissprot, KOG, Interpro and GO databases, respectively (Additional file 1: Figue S1). Moreover, a species distribution map was drawn (Fig. 3A). The results indicated that oats showed the most similar to \textit{Aegilops tauschii subsp. tauschii}, followed by \textit{Brachypodium distachyon} and \textit{Hordeum vulgare subsp. vulgare}.

4. The Analyses Of Differentially Expressed Genes (degs)

To obtain the differential expression genes response to high altitude, the Fragments Per Kilobase of transcript, FPKM method was used to analyze the expression abundance of unigenes. As a result, a total of 11,639 differentially expressed genes (log 2 Fold Change > 2) were differentially expressed between the low and the high altitudes (Additional file 2: Figue S2, Fig. 4A). Among them, 5,203 DEGs were found to be up-regulated and 6,436 DEGs were down-regulated (Fig. 4B). Interestingly, the number of down-regulation genes were more than the up-regulation ones. It indicated that more genes were down-regulated in response to high altitude stresses.

5. Go And Kegg Analyses Of Degs

To investigate the functions of these DEGs under high altitude stresses, we performed GO annotation enrichment analysis using the software package top GO (Additional file 3: Figue S3, Fig. 5A). The results showed that 2,879 transcripts were characterized and annotated with the top GO terms for classified genes being “cellular process” (1,956 transcripts) for biological processes. Other categories with high numbers of genes included “metabolic process” (1,819 transcripts) and “biological regulation” (887 transcripts). These showed that substance synthesis and catabolism, cellular processes and physiological regulation responses to external stresses were involved in responses of oats to high altitude environmental stresses. In addition, 3,308 transcripts were mapped to interior cell components in which “cell” (2,348 transcripts) and “cell part” (2,314 transcripts) were the most represented categories. The mechanism by which oats responding to high altitude was closely related to the structural composition of their cells, cell membranes and organelles. As for molecular functions, 4,011 transcripts were significantly annotated with the “binding” (2,533 transcripts) and “catalytic activity” (2,478 transcripts) pathways having the most DEGs, respectively. These indicated that the non-covalent bonding of molecules and the catalytic activity of enzymes in biochemical reactions played important roles in the physiological adaptability of oats to high altitude stresses.

At the same time, the DEGs and the biological processes involved in altitude stresses were illuminated and all of the DEGs were queried in KEGG databases. In this study 4,831 transcripts were allocated to 30 pathways in KEGG databases (Additional file 4: Figue S4, Fig. 5B) while “Biosynthesis of secondary metabolites” (1,060 transcripts), “plant-pathogen interaction” (541 transcripts) and “RNA transport” (504 transcripts) pathways were mostly enriched followed by “phenylpropanoid biosynthesis” (390 transcripts), “plant hormone signal transduction” (259 transcripts), MAPK sigaling pathway” (253 transcripts) and “starch and sucrose metabolism” (215 transcripts). Interestingly most of the genes
mapped in the first seven significantly enriched pathways had the trend in down-regulation. These results above indicated that various pathways were involved in responses of oats to different altitudes.

6. Analyses Of Transcription Factors (tf) In The Degs

TFs control the expression of numerous genes and play crucial functions in stress-induced signal transduction pathways. In this study, 125 TF genes were differentially expressed between two different altitudes (Additional file 5: Figure S5). Among them, 53 bHLH transcripts, 36 AP2-EREBP transcripts, 11 C2C2-CO-like transcripts were the top three most TF families. The rest of the transcription factors were ABI3VP1, C2C2-Dof, C2C2-GATA, bZIP, C2H2, ARF, ARR-B, BSD and C2C2-YABBY with the numbers of transcripts were 6, 6, 3, 3, 3, 1, 1, 1 and 1, respectively. These results indicated that the TFs above played critical roles in oats response to high altitude stresses.

7. Analyses of DEGs related to altitudinal responses

To further identify the key genes related to responses at different altitudes, the DEGs in two pathways for the chlorophyll metabolism and the carotenoid biosynthesis were compared in detail between the low and the high altitudes. The results demonstrated that 23 genes in the porphyrin and the chlorophyll metabolism pathways were detected. Among them, 14 genes had been up-regulated and only 9 genes were down-regulated at high altitude. The DEGs played important roles during the chlorophyll biosynthesis and encoding chlorophyllase (Additional file 6: Figure S6, Table 2). Moreover, the comparison of the DEGs involved in the carotenoid biosynthesis showed that 32 genes were significantly down-regulated at high altitude (Additional file 7: Figure S7). The results indicated that the photosynthetic pigment biosynthesis was an important process for oats under the high altitude stress.
### Table 2
Genotypic differences in expressions of DEGs associated with pigment metabolism.

| Group                      | Gene ID                  | Regulation | Gene description                          |
|----------------------------|--------------------------|------------|--------------------------------------------|
|                            | CL3626.Contig4_All       | up         | uroporphyrinogen decarboxylase             |
|                            | Unigene13229_All         | up         | coproporphyrinogen III oxidase             |
|                            | CL8939.Contig6_All       | up         | glutamyl-tRNA reductase                    |
|                            | CL9433.Contig7_All       | up         | glutamyl-tRNA synthetase                   |
| Chlorophyll and prophyrin  | CL900.Contig8_All        | up         | Glucuronosyltransferase                    |
| metabolism                | CL25634.Contig3_All      | up         | Glucuronosyltransferase                    |
|                            | CL8418.Contig1_All       | up         | protochlorophyllide reductase              |
|                            | CL22519.Contig2_All      | up         | chlorophyllase                             |
|                            | CL22519.Contig1_All      | up         | chlorophyllase                             |
|                            | CL5496.Contig2_All       | up         | chlorophyll(ide) b reductase               |
|                            | CL10250.Contig3_All      | up         | geranylgeranyl diphosphate                 |
|                            | CL10437.Contig1_All      | up         | geranylgeranyl diphosphate                 |
|                            | CL10437.Contig2_All      | up         | geranylgeranyl diphosphate                 |
|                            | CL23991.Contig6_All      | up         | cytochrome c oxidase assembly protein subunit 15 |
|                            | CL5813.Contig5_All       | down       | porphobilinogen synthase                   |
|                            | CL8939.Contig2_All       | down       | glutamyl-tRNA reductase                    |
|                            | CL9433.Contig14_All      | down       | glutamyl-tRNA synthetase                   |
|                            | Unigene4721_All          | down       | glucuronosyltransferase                    |
|                            | Unigene7840_All          | down       | glucuronosyltransferase                    |
|                            | Unigene16352_All         | down       | glucuronosyltransferase                    |
|                            | Unigene41659_All         | down       | geranylgeranyl-bacteriochlorophyllide a reductase |
|                            | Unigene36472_All         | down       | geranylgeranyl-bacteriochlorophyllide a reductase |
|                            | CL20435.Contig2_All      | down       | geranylgeranyl-bacteriochlorophyllide a reductase |

High altitude stresses might have an impact on plant photosynthesis. In this study, we identified 15 DEGs were significantly regulated in photosynthesis which played a part in photosystem I and II. Meanwhile, 9 genes that mapped to the photosynthesis-antenna protein pathways exhibited differential expressions at
high altitude, where the genes encoded light-harvesting complex II chlorophyll a/b binding protein (Additional file 8: Figure S8, Table 3). These results indicated that changes at the expression levels of the genes above might have blocked photosynthesis, thereby influencing the chlorophyll biosynthesis of oats at high altitude.
Table 3
Genotypic differences in expressions of DEGs associated with photosynthesis.

| Group             | Gene ID         | Regulation | Gene description                                      |
|-------------------|-----------------|------------|-------------------------------------------------------|
| Photosynthesis    | Unigene34665_All| up         | photosystem II CP47 chlorophyll apoprotein photosystem II CP47 chlorophyll apoprotein |
|                   | Unigene16087_All| up         | photosystem II oxygen-evolving enhancer protein 2      |
|                   | CL7675.Contig8_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | CL10733.Contig3_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | CL11120.Contig6_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | CL10733.Contig2_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | CL9061.Contig5_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | Unigene24290_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | CL25957.Contig11_All| up         | photosystem II oxygen-evolving enhancer protein 3      |
|                   | Unigene24271_All| up         | photosystem I P700 chlorophyll a apoprotein A1         |
|                   | CL399.Contig3_All| up         | photosystem I P700 chlorophyll a apoprotein A1         |
|                   | Unigene56697_All| up         | photosystem I P700 chlorophyll a apoprotein A1         |
|                   | Unigene13484_All| down       | photosystem I P700 chlorophyll a apoprotein A1/ A2     |
|                   | Unigene10050_All| down       | photosystem I P700 chlorophyll a apoprotein A1/ A2     |
|                   | CL10048.Contig11_All| down     | photosystem I subunit PsaO                            |
|                   |                 |            | ferredoxin                                            |
|                   |                 |            | photosystem II CP47 chlorophyll apoprotein             |
|                   |                 |            | photosystem II CP47 chlorophyll apoprotein             |
|                   |                 |            | ferredoxin–NADP + reductase                           |
| Group                              | Gene ID                     | Regulation | Gene description                                      |
|-----------------------------------|-----------------------------|------------|------------------------------------------------------|
| Photosynthesis-antenna protein    | Unigene13588_All            | up         | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | Unigene16609_All            | up         | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | Unigene23029_All            | up         | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | Unigene15222_All            | up         | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | CL25362.Contig4_All         | up         | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | Unigene14215_All            | up         | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | Unigene21557_All            | down       | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | Unigene75195_All            | down       | light-harvesting complex II chlorophyll a/b binding protein 1 |
|                                   | CL23904.Contig9_All         | down       | light-harvesting complex II chlorophyll a/b binding protein 1 |

Hormones are pivotal to plants in stress adaptive signaling cascades and act as central integrators to connect and reprogram different responses. In this experiment, we identified 273 hormone-related DEGs between the low and the high altitude conditions. Among them, 60 genes were identified to encode DELLA protein and acted as a part of gibberellin receptor and phytochrome-interacting factor. Forty-eight genes were identified in the auxin signal transduction pathway in which some encoded auxin-responsive protein IAA and others participated in the process of auxin synthesis and metabolism. In jasmonic acid signal transduction pathway, 41 genes encoded jasmonate ZIM domain-containing protein and transcription factor MYC2. The three pathways above were the most abundant phytohormone signal transduction pathways. In addition, 33 genes in cytokinin pathway, 31 genes in abscisic acid signal pathway, 24 genes in brassinosteroid pathway, 22 genes in ethylene pathway and 14 genes in salicylic acid pathway were differentially expressed (Additional file 9: Figue S9). The results above suggested that these hormone-related genes can be involved in cell division, stem elongation, stomata aperture, fruit maturation and disease prevention processes in response to high altitude conditions.

8. Relative Degs Measured By Qrt-pcr

To further verify the accuracy of RNA-seq data and confirm the level of differential expression genes, we performed randomly qRT-PCR from ten genes induced by the high altitude and concluded two chlorophyll metabolism pathways and eight hormone-related DEGs as was shown in Table 2 and Additional file 10. The results revealed that the ten DEGs were up-regulated in accordance with the results of the RNA-seq
analysis Fig. 6. The results suggested that RNA-Seq data be reliable and the above genes were involved in high altitude stresses.

**Discussion**

The Qinghai-Tibet Plateau itself has unique climate and geographical features which pose huge challenges to the survival of plants. Although plants cannot escape from unfavorable environments, they can change their morphological structures, physiological and molecular processes in which changes happen in endogenous secondary metabolite levels, ion homeostasis, gene expressions and protein activity regulations to adapt to different environmental conditions. Oats are an important source of food and feed in the Qinghai-Tibet Plateau and are beneficial to human health because they are rich in protein and minerals[18]. Now the data available on the molecular basis of oats response to Qinghai-Tibet Plateau conditions stress is not limited any longer with the development of high-throughput sequencing.

1. **The adaptation of the oat physiological characteristics to high altitude environments**

The increased levels of protective antioxidant enzymes could maintain the homeostasis of ROS. In this study, oats could regulate many physiological processes to adapt to high altitude stresses, e.g. increasing the activities of SOD (Fig. 1B) which suggested that oats can improve their ability to resist damages by establishing a better antioxidant enzyme system under high altitude stresses. The membrane lipid peroxidation product MDA was also one of the important indicators of cell damages which could bind to proteins and cause membrane protein denaturation [19]. Our result showed that oats suffered more severe damages to the cell membrane and antioxidant system in high altitude environments than those at low altitude (Fig. 1A). In an alpine environment, low temperatures could affect the osmotic pressure of plant cells. As shown in Fig. 1C, high altitude stresses induced the accumulation of soluble sugars in oats. Therefore, soluble sugars might play important roles in oat growth and development and responses to abiotic stresses, and eventually affect yield and quality. In addition, sugars acted as not only an essential source of carbon but also signaling molecules in response to the integration of information from environments as well as developmental and metabolic cues in plants [20–22]. Therefore, it could further improve the resistance of oats under low temperatures at high altitude by increasing the content of soluble sugars to avoid protein coagulation. Meanwhile, oat plants showed sharp decreases in chlorophyll content and NPQ at high altitude (Fig. 1D, E). The decreases of chlorophyll content might be because the enhancement of radiation could exacerbate the photooxidation process of photosynthetic pigments or restrain some synthetic processes to decrease the light energy absorbed by leaves at high altitude to avoid or reduce the radiation damages on plants [23]. The decrease in NPQ indicated that the plant did not dissipate excess excitation energy in the form of heat, thereby increased the damage on PSII by the accumulation of the excitation energy under high altitude stresses [24]. Moreover, the chlorophyll content and NPQ as photosynthesis markers were closely linked to yield which was probably ascribed to the reductions of both plant height and the ratio of stem/leaf, subsequently led to marked decreases in hay yield index (Fig. 2). In addition, as was shown on Fig. 2 high altitude conditions could regulate the development of oat plants, e.g. decreased the stomatal density to reduce water loss to adapt to high
altitude stress [25]. This might be due to the water shortage caused by low temperatures which was also the result of adaptation to low temperature and strong radiation in high mountains.

When subjected to abiotic stresses, TFs could play an important role by binding to cis-regulated DNA elements in gene promoters and activating or inhibiting their expressions[26]. bHLH had been reported to directly bond the phytochrome action factor in the photoreceptor signaling network which specifically bound to phytochrome responsive gene promoter G-box regulatory elements in vitro[27]. In addition, overexpression of Arabidopsis bHLH TFs enhanced chilling or osmotic tolerance by reducing malondialdehyde content and accumulating ROS[28, 29]. AP2-EREBP and ABI3 TF could participate in the process of plant growth, development and respond to stresses by regulating the expression of downstream target genes[30, 31]. In our study, bHLH, AP2-EREBP and ABI3VP1 were the three most abundant transcription factors identified as DEGs in response to high altitude stresses. Moreover, many transcription factors containing C2C2 zinc finger domain were found including C2C2-CO-like, C2C2-Dof, C2C2-GATA and C2C2-YABBY. Researches had shown that they played vital biological functions in vegetative tissue development and multiple stress responses [32]. It could be inferred that bHLH, AP2-EREBP, ABI3VP1 and C2C2 TFs might play important roles in response to the high altitude stress. In addition, many other TF families were also identified to be differentially expressed such as bZIP, C2H2, ARF, ARR and BSD. This also explained that numerous TFs participated actively in the regulation of oats in response to high altitude conditions through different pathways.

3. High altitude affected the expression of genes related to pigment metabolism and photosynthesis

Plant pigments determine color variations of leaves which are complex biological processes. Among them, chlorophyll and carotenoid are two important antenna pigments in the photosynthetic system. Chlorophyll plays a central role in the process of absorbing and transmitting energy [33]. Carotenoids can protect chlorophyll from degradation as a major component of the photosynthetic apparatus and photoprotection system [38]. Plateau plants can adapt to UV-B radiation by changing the content of photosynthetic pigments in leaves such as reducing the chlorophyll and carotenoid content [34]. Chlorophyll and carotenoid metabolisms are complex processes involving multiple enzymes. Comparative transcriptome profiling data for the low altitude and the high altitude showed that the expression of numbers of DEGs were significantly regulated including uroporphyrinogen decarboxylase, coproporphyrinogen III oxidase, protochlorophyllide reductase, chlorophyll(ide) b reductase and cytochrome c oxidase assembly protein subunit 15, which were key enzymes associated with the process of chlorophyll synthesis [35]. Meanwhile, our comparison of the DEGs involved in carotenoid biosynthesis showed that 32 genes were significantly down-regulated in high altitude (Additional file 3: Figure S3). It might imply that oats can adapt to UV-B radiation stress by changing the content of chlorophyll and carotenoids in leaves.

Chloroplasts are the main sites for photosynthesis which are comprised of membranes, thylakoid system, and stroma with functional units in it. In higher plants, the multi-subunit pigment–protein complexes (i.e., photosystem (PS) I, PSII, light harvesting complexes, cytochrome b6/f, and ATP synthase) are embedded
in the highly folded thylakoid membrane where they are responsible for light absorption and energy transfer [36]. Strong radiation in high-altitude environments might have been related to thylakoid development and photosynthesis at the two different altitudes. Based on transcriptome data it was found that genes encoding photosystem II CP47 chlorophyll apoprotein, photosystem II oxygen-evolving enhancer protein, photosystem I P700 chlorophyll a apoprotein and photosystem I subunit PsaO were significantly regulated at high altitude. In higher plants, light-harvesting complex II chlorophyll a/b binding proteins that accumulate in the thylakoid membranes, where their proposed function is in photoprotection [37–39]. The chlorophyll a/b-binding proteins are correlated with photodamage in the PSII reaction centers [40]. We found that the proteins were significantly enriched suggesting that strong photosynthetic apparatus damage occur in high altitude (Table 3). These results were consistent with our previous photosynthetic physiological index analysis (Fig. 2B) which further demonstrated that the genes related to pigment metabolism and photosynthesis in oats could respond to high altitude stresses.

4. Identification of signal transduction genes responsible for high altitude stress

The different expression of genes involved in the signal cascade mechanism can affect the expression of genes participating in the formation of plant hormones such as auxin, ABA, ET, SA and JA. Theses hormones may amplify the cascades or initiate some new signaling pathways[41, 42]. It is now known that these phytohormones extensively regulate all aspects of plant stress responses ranging from signal cascade transduction to modifications in plant developmental processes[43]. In this experiment, GA, IAA and JA were the top three responsive hormone signal transduction pathways in oats and there were a number of DEGs involved in them. Studies had shown that the gibberellin receptor sensing the GA signal could activate the signaling pathway, thereby regulating the expression of downstream genes to affect plant growth and morphogenesis[44]. Totally 60 DEGs involved in the response to GA signal transduction pathway were identified as being differentially expressed, which encoded gibberellin receptor GID and DELLA proteins. This indicated that high altitude-induced expression of oats appeared to be GA dependent.

Auxin is a plant hormone extensively involved in regulating cell growth, cell division and cell-specific differentiations to participate in a series of growth and development processes [45]. ARF and SAUR family protein could bind to the cis-acting element of auxin-inducible gene and regulate its transcriptional expression[46, 47]. Forty-eight DEGs encoding three key proteins (transport inhibitor response, auxin-responsive and SAUR family protein) of IAA signaling pathway were identified in our study. It could infer that both ABF and SAUR can activate the down-stream gene expressions to respond to high altitude stresses.

JAs are a group of important hormones that play pivotal roles in a variety of plant growth, developmental and stress response processes[48]. In this study, 41 DEGs encoding jasmonate ZIM domain-containing protein and transcription factor MYC2 were enriched in the JA signaling pathway. It had been shown that JAZ gene and MYC2 were the major regulators of jasmonic acid signaling pathway in which JAZ protein
degradation could release MYC2 transcription factor and activate the expression of downstream genes of jasmonic acid signaling pathway [49].

**Conclusion**

In order to understand the mechanisms of oats adapting to high altitudes, we accomplished physiological and agronomical characterizations and transcriptome sequence analysis. The physiological and agronomical results showed that oats displayed significant increases in MDA, SOD and soluble sugar contents, accompanied by decreases in chlorophyll, NPQ, stomatal density and hay yield. The transcriptomic profiles showed apparent differences between low and high altitudes where many pathways associated with high altitude stresses were identified including photosynthetic pigment synthesis, photosynthesis, and plant hormone signaling transduction pathways. Moreover, large amount of differentially expressed TFs between low and high altitudes was identified to participate in the response to altitude stress of oats, which can serve as candidate genes for altitude stresses molecular breeding project of oat. In general, this results provided the basis for understanding better the biological regulation on oats under two scenarios of altitudes and thus contributed to breeding efforts aimed at increasing yield under such situations.

**Methods**

1. **Plant Materials And Growth Conditions**

   Seeds of Avena sativa L. Qingyin No.1 were collected from the Qinghai with the permission of the Qinghai Academy of Animal Science and Veterinary Medicine in the Qinghai Province of China. Animal Science and Veterinary Medicine in the Qinghai Province of China undertook the formal identification of the samples and provided details of specimen deposited. In order to investigate the effects of altitudes on the physiology and the transcriptions, oat leaf samples of the cultivar (cv.) "Qingyin No.1" were collected at heating stage in April, 2015 in two regions with significant differences in altitude in Qinghai Province, China, County of Minhe, (36°14′, 102°41′) at the altitude ca. 2080 m (defined as the low altitude) and County of Huangzhong (36°21′, 101°44′) at ca. 2918 m (defined as the high altitude). During the heating period, the climate data in the two altitude regions were recorded (Additional file 10: Figue S10). The field plots were arranged as a completely randomized block design and adopted line sowing method. The distance of row spacing and seeding depth of were 20 cm and 5 cm, respectively. The experiment was carried out without fertilization and irrigation and weeding manually. The whole flag leaf was collected randomly and immediately frozen in liquid nitrogen. Samples were kept at −80°C for further analyses.

2. **The Measurements Of Agronomic Traits**

   Plant heights were measured at heading stage of oats. Each of the 5 oat plants in each plot was selected randomly to measure its height to calculate their mean. At the same stage, the ratio of stem to leaf was measured. About 0.5 kg of oat samples were weighed in each plot, and stems, leaves and inflorescences
were separated into two parts. After air dried, samples were weighed and the ratio of stem to leaf was calculated (repeated with above). The leaf sheath of the oat grasses was collected into stems and the ears into leaves. The fresh grass samples were naturally air-dried for the dry weights which were converted into hay yield per hectare. After weighed, the hay samples were crushed and sieved with 40-mesh then the crude fat (EE) was determined by the soxhlet extraction.

3. Measurements Of The Stomatal Density And Physiological Analyses

The stomatal density of oats grown at the two altitudes were observed by SEM.

SOD activity was detected by recording the decrease in optical density of NBT dye by the enzyme. MAD content was measured using a modified TBA method. The two methods above were described by Tang et al.

Approximate 0.1 g of each leaf sample was incubated on ice for 30 min and immersed in 10 ml of 95% ethanol in 25 ml brown flask at room temperature. Until the green color disappeared, the solution was set to volume, shaken and stored in the dark. A series of 100 µl aliquots were taken every 10 min after initial immersion, and subjected to spectrophotometry (absorption measured at 647 nm and 664 nm) to quantify the amount of chlorophyll leached.

Before Fv/Fm measurement, whole oat plants were dark-adapted for 20 min, and Fv/Fm was measured using a steady-state gas-exchange system with an integrated fluorescence chamber head (Heinz Walz GmbH, Effeltrich, Germany). Fv/Fm values were calculated as Fv/Fm = (Fm – Fo)/Fm, NPQ = Fm /Fm.’

4. Library Preparation For Transcriptome Sequencing

RNA extraction was performed according to the manufacturer's protocol of the RNA prep Pure Plant kit (KangWei Biotech Co. Ltd., Beijing, China). RNA purity and integrality were checked before the cDNA library construction. The mRNA was enriched from the total RNA through the magnetic beads with oligo (dT). Afterward, mRNA was randomly broken into fragments. Catalyzed by reverse transcriptase, double-stranded cDNA was synthesized through mRNA by combining with random primers. After ds-cDNA purified, the adapters and poly (A) tails were ligated at both ends of ds-cDNA. With the adapter sequences, ds-cDNA fragments were amplified through PCR. After ds-cDNA quantification, the cDNA libraries were ready for sequencing using the Illumina HiSeq Xten (single strands for sequencing).

5. Transcriptome Assembly And Gene Function Annotations

The clean data were obtained by removing reads containing adapters and low quality ones from raw data. Transcriptome assemblies were performed using Trinity[50] based on clean data. The sequences of unigenes were compared with Nr (NCBI non-redundant), Nt (Nucleotide Sequence Database), SwissProt (a manually annotated and reviewed protein sequence database) and GO (Gene Ontology), KOG (euKaryotic Orthologous Groups) databases by BLAST and the KEGG (Kyoto
Encyclopedia of Genes and Genomes). Orthology results were obtained by comparing with KEGG using KOBAS2.0[51].

6. Differential Expression Analyses

The EdgeR program package was used to adjust read counts of each sequenced library and the RSEM package for differential expression level analyses of two samples. The p value was adjusted using the q value. In this study, q value < 0.01 and |log2 (fold change)| >2 were set as the thresholds for significantly differential expressions.

Abbreviations

NPQ: non-photochemical quenching coefficient; DEGs: differentially expressed genes; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; Nr: NCBI non-redundant; Nt: Nucleotide Sequence Database; SwissProt: a manually annotated and reviewed protein sequence database; KOG: euKaryotic Orthologous Groups; TFs: transcription factors; Chl a: chlorophyll a; APX: ascorbate oxidase; CAT: catalase; GR: glutathione reductase; MDA: Malondialdehyde; SOD: superoxide dismutase; ROS: Reactive oxygen species; qRT-PCR: Quantification real-time polymerase chain reaction; RNA-seq: RNA sequencing; SEM: scanning electron microscopy; IAA: Auxin; ABA: Abscisic acid; ET: Ethylene; SA: Salicylic acid; JA: Jasmonic acid; ARF: Auxin response factor; NBT: nitro-blue tetrazolium; TBA: thiobarbituric acid; FPKM: per Million mapped reads; RSEM: RNA sequencing by expectation maximization

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequencing data have been submitted to the NCBI Sequence Read Archive database with accession number PRJNA639320.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding
The RNAseq analysis was supported by National Natural Science Foundation of China (31872998). The funding body had no influence on the experimental design, data analysis and interpretation, or writing of the manuscript.

Authors’ contributions

JQY, GWC and YJC conceived and designed the experiments. JQY, BL, WHH and LL performed the experiments. JQY, BL, TTS, JLH and ZLKL analyzed and interpreted the sequence data. JQY wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank the Biomarker Technologies (Beijing, China) for the RNA-seq and for the raw data analysis.

References

1. Qiao Q, et al. Transcriptome sequencing of Crucihimalaya himalaica (Brassicaceae) reveals how Arabidopsis close relative adapt to the Qinghai-Tibet Plateau. Sci Rep. 2015;6:21729.
2. Chi X, et al. Genetic Structure and Eco-Geographical Differentiation of Lancea tibetica in the Qinghai-Tibetan Plateau. Genes (Basel). 2019;10:97.
3. Jia Y, et al. Comparative Transcriptome Analysis Reveals Adaptive Evolution of Notopterygium incisum and Notopterygium franchetii, Two High-Alpine Herbal Species Endemic to China. Molecules. 2017;22:1158.
4. Xin GS, et al. Blood mineral status of grazing Tibetan sheep in the Northeast of the Qinghai–Tibetan Plateau. Livestock Science. 2011;136:102-107.
5. Storz JF, Scott GR, Cheviron ZA. Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. J Exp Biol. 2010;213:4125-4136.
6. Yang Y, et al. Comparative transcriptomic analysis revealed adaptation mechanism of Phrynocephalus erythrurus, the highest altitude Lizard living in the Qinghai-Tibet Plateau. BMC Evol Biol. 2015;15:101.
7. Thiebaut F, Hemerly AS, Ferreira PCG. A Role for Epigenetic Regulation in the Adaptation and Stress Responses of Non-model Plants. Front Plant Sci. 2019;10:246.
8. Zhu Pj, Yang L. Ambient UV-B radiation inhibits the growth and physiology of Brassica napus L. on the Qinghai-Tibetan plateau. Field Crops Research. 2015;171:79-85.
9. Li X, et al. Comparative proteomics analyses of Kobresia pygmaea adaptation to environment along an elevational gradient on the central Tibetan Plateau. PLoS One. 2014;9:e98410.
10. Foyer CH. Redox Homeostasis and Antioxidant Signaling: A Metabolic Interface between Stress Perception and Physiological Responses. The Plant Cell. 2005;17:1866-1875.
11. Vinocur B. Altman A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol. 2005;16:123-132.
12. Ma L, et al. Physiological, biochemical and proteomics analysis reveals the adaptation strategies of the alpine plant Potentilla saundersiana at altitude gradient of the Northwestern Tibetan Plateau. Journal of Proteomics. 2015;112:62-83.

13. Ma L, et al. Comparative proteomic analysis reveals the role of hydrogen sulfide in the adaptation of the alpine plant Lamiophlomis rotata to altitude gradient in the Northern Tibetan Plateau. Planta. 2015;241:887-906.

14. Zhao SY, et al. Genetic Adaptation of Giant Lobelias (Lobelia aberdarica and Lobelia telekii) to Different Altitudes in East African Mountains. Front Plant Sci. 2016;7:488.

15. Sharma P, et al. Polymorphism analysis in advanced mutant population of oat (Avena sativa L.) using ISSR markers. Physiol Mol Biol Plants. 2016;22:115-120.

16. Anwar A, et al. Biological Roles of Ornithine Aminotransferase (OAT) in Plant Stress Tolerance: Present Progress and Future Perspectives. Int J Mol Sci. 2018;19:3681.

17. Wu B, et al. Transcriptome analysis of hexaploid hulless oat in response to salinity stress. PLoS One. 2017;12:e0171451.

18. Noga A, et al. Conversion of oat (Avena sativa L.) haploid embryos into plants in relation to embryo developmental stage and regeneration media. In Vitro Cell Dev Biol Plant. 2016;52:590-597.

19. Yang HL, et al. Antihemolytic and antioxidant properties of pearl powder against 2,2′-azobis(2-amidinopropane) dihydrochloride-induced hemolysis and oxidative damage to erythrocyte membrane lipids and proteins. J Food Drug Anal. 2017;25:898-907.

20. Ma QJ, et al. Transcription Factor AREB2 Is Involved in Soluble Sugar Accumulation by Activating Sugar Transporter and Amylase Genes. Plant Physiol. 2017;174:2348-2362.

21. Lastdrager J, Hanson J, Smeekens S. Sugar signals and the control of plant growth and development. J Exp Bot. 2014;65:799-807.

22. Lilley JL, et al. An endogenous carbon-sensing pathway triggers increased auxin flux and hypocotyl elongation. Plant Physiol. 2012;160:2261-2270.

23. Lizana XC, Hess S, Calderini DF. Crop phenology modifies wheat responses to increased UV-B radiation. Agricultural and Forest Meteorology. 2009;149:1964-1974.

24. COSTA ES. Chlorophyll a fluorescence analysis in response to excitation irradiance in bean plants (Phaseolus vulgaris L. and Vigna unguiculata L. Walp) submitted to high temperature stress. PHOTOSYNTHETICA. 2003;41:77-82.

25. Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys. 2005;444:139-158.

26. Singh D, Laxmi A. Transcriptional regulation of drought response: a tortuous network of transcriptional factors. Front Plant Sci. 2015;6:895.

27. Huq E, et al. PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. The EMBO Journal. 2002;21:2441-2450.
28. Zhao Q, et al. Tobacco Transcription Factor NtbHLH123 Confers Tolerance to Cold Stress by Regulating the NtCBF Pathway and Reactive Oxygen Species Homeostasis. Front Plant Sci. 2018;9:381.

29. Liu W, et al. bHLH122 is important for drought and osmotic stress resistance in Arabidopsis and in the repression of ABA catabolism. New Phytol. 2014;201:1192-1204.

30. Liu C, Zhang T. Expansion and stress responses of the AP2/EREBP superfamily in cotton. BMC Genomics. 2017;18:118.

31. Zhao M, et al. Regulatory Mechanism of ABA and ABI3 on Vegetative Development in the Moss Physcomitrella patens. Int J Mol Sci. 2018;19:2728.

32. Ge Y, et al. Alkaline-stress response in Glycine soja leaf identifies specific transcription factors and ABA-mediated signaling factors. Funct Integr Genomics. 2011;11:369-379.

33. Tanaka A, Tanaka R. Chlorophyll metabolism. Curr Opin Plant Biol. 2006;9:248-255.

34. Sicora C, et al. UV-B and UV-A Radiation Effects on Photosynthesis at the Molecular Level. Earth and Environmental Sciences. 2006;57:121-135.

35. Mazor Y, Borovikova A, Nelson N. The structure of plant photosystem I super-complex at 2.8 A resolution. Elife. 2015;4:e07433.

36. BASHIR H, et al. Chloroplast and photosystems: Impact of cadmium and iron deficiency. Trends Plant Sci. 2011;53:321-335.

37. Hutin C, et al. Early light-induced proteins protect Arabidopsis from photooxidative stress. PNAS. 2003;100:4921-4926.

38. Beck J, et al. Small One-Helix Proteins Are Essential for Photosynthesis in Arabidopsis. Front Plant Sci. 2017;8:7.

39. Ozawa SI, et al. Configuration of Ten Light-Harvesting Chlorophyll a/b Complex I Subunits in Chlamydomonas reinhardtii Photosystem I. Plant Physiol. 2018;178:583-595.

40. Heddad M, et al. Differential expression and localization of early light-induced proteins in Arabidopsis. Plant Physiol. 2006;142:75-87.

41. Sarwat M, et al. Phytohormones and microRNAs as sensors and regulators of leaf senescence: assigning macro roles to small molecules. Biotechnol Adv. 2013;31:1153-1171.

42. Wang K, et al. Transcriptome analysis of chrysanthemum (Dendranthema grandiflorum) in response to low temperature stress. BMC Genomics. 2018;19:319.

43. ShuY, et al. Transcriptome sequencing and expression profiling of genes involved in the response to abiotic stress in Medicago ruthenica. Genet Mol Biol. 2018;41:638-648.

44. Ito T, et al. DELLA-dependent and -independent gibberellin signaling. Plant Signal Behav. 2018;13:e1445933.

45. Tuan PA, et al. Transcriptomics of cytokinin and auxin metabolism and signaling genes during seed maturation in dormant and non-dormant wheat genotypes. Sci Rep. 2019;9:3983.
46. Spartz AK, et al. SAUR Inhibition of PP2C-D Phosphatases Activates Plasma Membrane H+-ATPases to Promote Cell Expansion in Arabidopsis. Plant Cell. 2014;26:2129-2142.

47. Zhang Y, et al. Genome-wide characterization of the auxin response factor (ARF) gene family of litchi (Litchi chinensis Sonn.): phylogenetic analysis, miRNA regulation and expression changes during fruit abscission. PeerJ. 2019;7:e6677.

48. Sharma M, Laxmi A. Jasmonates: Emerging Players in Controlling Temperature Stress Tolerance. Front Plant Sci. 2015;6:1129.

49. Boter M, et al. Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev. 2004;18:1577-91.

50. Grabherr MG, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29:644-652.

51. Altschul SF, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research. 1997;25:3389-3402.

Additional Files

Additional file 1: Figure S1. Function annotation results of oat.

Additional file 2: Figure S2. Annotation of 11,639 significantly differentially-expressed genes transcripts.

Additional file 3: Figure S3. Gene Ontology (GO) functional annotation of transcripts.

Additional file 4: Figure S4. KEGG pathway mapping

Additional file 5: Figure S5. Genotypic differences in expressions of DEGs associated with TFs between low and high altitude.

Additional file 6: Figure S6. Differently-expressed genes related to porphyrin and chlorophyll metabolism.

Additional file 7: Figure S7. Differently-expressed genes related to carotenoid biosynthesis.

Additional file 8: Figure S8. Differently-expressed genes related to photosynthesis.

Additional file 9: Figure S9. Differently-expressed genes related to hormone signal transduction pathway.

Additional file 10: Figure S10. Meteorological data of the growing season of Minhe and Huangzhong County (April-October 2015).

Figures
Figure 1

Physiological changes of oats at low and high altitudes. (A) MDA activity, (B) SOD activity, (C) chlorophyll content, (D) Fv/Fm, (E) NPQ, (F) stomata density. Values represented the means (±SE) from three fully independent biological replicates. Significant differences from the low altitude were denoted by one or two asterisks corresponding to p < 0.05 and p < 0.01, respectively, by the Student’s t test. (G) The photographs of stomata of oats at low and high altitudes.
Figure 2

Agronomic trait changes of oats at low and high altitudes. (A) plant height, (B) crude protein, (C) crude fat, (D) hay yield. Means were compared using one-way ANOVA. All parameters were shown as the mean±SE. Significant differences from low altitude were denoted by one or two asterisks corresponding to p < 0.05 and p < 0.01, respectively by the Student’s t test.

Figure 3

Functional annotations of the assembled transcriptome. (A) Similarities of Oats to other species.
Figure 4

Venn diagram and volcano plot of DEGs at low and high altitudes. (A) Venn diagram showed DEGs expressed at each of the two altitude treatments. (B) Volcano plot showed the numbers of DEGs identified in comparisons between pairs of libraries.
Figure 5
Functional annotation of assembled transcriptome. (A) Gene ontology (GO) classifications of the annotated DEGs (up- and down-regulated). (B) Kyoto Encyclopedia of Genetics and Genomics (KEGG) database analyses of DEGs (up- and down-regulated) enriched in different biological pathways. The X-axis represented enriched pathways and Y-axis represented the total number of transcripts.

Figure 6

The relative expression levels of DEGs identified in the comparison between RNA-Seq and qRT-PCR. The gene relative expression levels were determined by $2^{-\Delta\Delta CT}$ as expressed, and were normalized to the expression level of UBC. Error bars represented the standard deviations of three PCR replicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile10.xlsx
- Additionalfile9.xlsx
- Additionalfile8.xlsx
- Additionalfile7.xlsx
- Additionalfile6.xlsx
- Additionalfile5.xlsx
- Additionalfile4.xlsx
- Additionalfile3.xlsx
- Additionalfile1.xlsx
- Additionalfile2.xlsx