# Physiological and transcriptomic analyses reveal novel insights into the cultivar-specific response to alkaline stress in alfalfa (*Medicago sativa* L.)

**Tian-Jiao Wei** ( tianjiao25@126.com )
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences https://orcid.org/0000-0002-2550-5609
**Guang Li**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences
**Ming-Ming Wang**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences
**Yang-Yang Jin**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences
**Guo-Hui Zhang**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences
**Miao Liu**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences
**Hao-Yu Yang**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences
**Chang-Jie Jiang**
National Agriculture and Food Research Organization Institute of Agrobiological Sciences: Nogyo Shokuhin Sangyo Gijutsu Sogo Kenkyu Kiko Seibutsu Kino Riyo Kenkyu Bumon
**Zheng-Wei Liang**
Northeast Institute of Geography and Agroecology Chinese Academy of Sciences https://orcid.org/0000-0002-0309-3463

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# Abstract

***Key message*** Candidate pathways for alkaline tolerance in alfalfa seedlings were identified; these included those for homeostasis of ions and redox status, biosynthesis of phenylpropanoids, flavonoids, and amino acids, and MAPK signaling.

**Abstract** Soil alkalization severely limits plant growth and development; however, the mechanisms of alkaline response remain largely unknown. In this study, we performed physiological and transcriptomic analyses using two alfalfa cultivars (*Medicago sativa* L.) with different sensitivities to alkaline conditions. The chlorophyll content and shoot fresh weight drastically declined in the alkaline-sensitive cultivar Algonquin (AG) following alkaline treatment (0-25 mM $Na_2CO_3$ solution), while the alkaline-tolerant cultivar Gongnong NO.1 (GN) maintained relatively stable growth and chlorophyll content. Physiological analysis revealed that compared with AG, GN had higher contents of $Ca^{2+}$ and $Mg^{2+}$; the ratios of $Ca^{2+}$ and $Mg^{2+}$ to $Na^{+}$, proline and soluble sugar, and enzyme activities of peroxidase (POD) and catalase (CAT) decreased under the alkaline conditions. Further, transcriptomic analysis identified three categories of alkaline-responsive differentially expressed genes (DEGs) between the two cultivars: 48 genes commonly induced in both the cultivars (CAR), 574 genes from the tolerant cultivar (TAR), and 493 genes from the sensitive cultivar (SAR). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses showed that CAR genes were mostly involved in phenylpropanoid biosynthesis, lipid metabolism, and DNA replication and repair; TAR genes were significantly enriched in metabolic pathways, biosynthesis of secondary metabolites, MAPK signaling pathway, and flavonoid and amino acid biosynthesis; the SAR genes were specifically enriched in vitamin B6 metabolism. Taken together, the results identified candidate pathways associated with genetic variation in response to alkaline stress, providing novel insights into the mechanisms underlying alkaline tolerance in alfalfa.


# Introduction

Soil salinization and alkalization are important ecological and agricultural problems that seriously inhibit plant growth and limit worldwide crop yields (Julkowska and Testerink 2015). More than 830 million ha of land suffer from salinization and/or alkalization (Krasensky and Jonak 2012). As one of China's five largest salt-affected soil regions, the Songnen Plain located in the northeast of China has 3.78 million ha of saline-alkaline (SA) land (Yang et al. 2016). Compared to neutral salt stress (NaCl), the SA stress occurs in more diversified forms and comprises high $Na^{+}$ contents and high $CO_3^{2-}$ or $HCO_3^{-}$ contents (high pH), resulting in more damaging effects to plants (An et al. 2016; Long et al. 2013; Peng et al. 2008). Understanding the different mechanisms by which plants perceive and react to such environmental stresses should lead to novel strategies for crop improvement.

When $Na^{+}$ accumulates to high levels in plant cells, adverse effects, including $Na^{+}$ toxicity, osmotic shock, and oxidative stress, occur (An et al. 2016; Parida and Das 2005; Zhang et al. 2017). $Na^{+}$ entry into cells triggers a battery of signaling pathways, such as $Ca^{2+}$ and reactive oxygen species (ROS), ultimately leading to a reprograming of the gene expression profile to maintain homeostasis at the molecular and physiological levels (Choudhury et al. 2017; Zhu 2016). In addition to high salinity, SA stress is also characterized by high pH that destroys the integrity of root cell membranes (Ding et al. 2019; Zhang et al. 2017), disrupts the root ion homeostasis (Tester and Davenport 2003), and reduces photosynthesis and photosynthetic electron transport (An et al. 2016; Li et al. 2010). To cope with these stresses, plants have evolved molecular, physiological, and ecological strategies for either avoiding or tolerating the stresses, including accumulating inorganic ions (Ashrafi et al. 2018; Yang et al. 2007) and osmotic substances to reduce osmotic damage (Ashrafi et al. 2014; Li et al. 2010; Rokebul et al. 2017) and activating antioxidant enzymes to scavenge the excessive ROS (Apel and Hirt 2004; Zhang et al. 2017).

Alfalfa (*Medicago sativa* L.) is a type of capital forage due to its high yield and good quality (Scasta et al. 2012), and it has relatively stronger salt tolerance than other species (Munns and Tester 2008). Moreover, alfalfa can improve the soil environment owing to its ability to reduce soil salinity and fix nitrogen through *Rhizobium* microbes (Moses et al. 2018). Alfalfa is widely cultivated in SA arable land in North China (An et al. 2016; Long et al. 2019). However, the local soil is at risk of SA stress that would seriously affect the productivity of alfalfa (An et al. 2016). Therefore, elucidating the molecular mechanism of SA tolerance of alfalfa is the motivation for cultivating tolerant cultivars in SA land. Although recent studies have documented the physiological response to SA stress in rice (Liu et al. 2019), sorghum (Sun et al. 2019), wheat (Guo et al. 2015), tomato (Wang et al. 2015) and corn (Guo et al. 2017), there is relatively little information concerning the molecular mechanism of SA tolerance in alfalfa.

With the rapid development of analytical technology, increasing numbers of researchers have begun to apply high-throughput sequencing to study saline tolerance or SA tolerance mechanisms of alfalfa. Some salt tolerance genes (Lei et al. 2018), key osmotic and ionic stress-related genes (Luo et al. 2019), and transcription factors related to salt stress have been identified (Postnikova et al. 2013). An et al. (2016) emphasized that anti-oxidation and detoxification play important roles in the SA tolerance of alfalfa. Long et al. (2019) found that some alkaline-responsive proteins influence diverse processes such as catalytic activity, signaling, and antioxidant activity. Furthermore, several lines of evidence have strengthened the hypothesis that saline tolerance of the plant has a tissue-specific response; the leaves are considered to respond to saline stress more sensitively than roots (Lei et al. 2018; Munns and Tester 2008). This occurs mainly through compartmentalizing excessive cytosolic $Na^+$ into vacuoles, or the excretion of $Na^+$ via special structures such as salt glands (Lei et al. 2018; Munns and Tester 2008; Yuan et al. 2015). Although several observations have been made for the whole plant (An et al. 2016; Luo et al. 2019), the physiological and molecular mechanisms underlying alfalfa's response to SA stress remain largely elusive.

This study aimed to explore the physiological and molecular responses to alkaline stress using two alfalfa cultivars with different sensitivities to alkaline stress. We show that pathways involved in homeostasis of ions and redox status, biosynthesis of phenylpropanoids, flavonoids, and amino acids, and MAPK activation are important for alkaline tolerance in alfalfa seedlings.

# Materials And Methods

### Plant material and alkaline-stress treatment

An alkaline-tolerant cultivar, Gongnong NO.1 (GN), and an alkaline-sensitive cultivar, Algonquin (AG), were selected based on our previous study (Wei et al. 2020). The seeds were sterilized with a solution containing 6% sodium hypochlorite for 10 min and rinsed with distilled water three times. Fifteen seeds were sown in plastic pots (25 cm diameter × 20 cm depth) filled with sand, and seedlings were cultured in a controlled growth chamber under the following conditions: 25°C day/20°C night and 12-h photoperiods under light intensity of approximately 350 μmol photons $m^{-2}$ $s^{-1}$. The pots were irrigated with distilled water for 14 days until the first trifoliate leaf stage, and then supplied with Hoagland solution every day for another 10 days.

To determine the proper treatment concentration of $Na_2CO_3$, 25-day-old alfalfa seedlings (approximately the third trifoliate leaf stage) were separately treated with solutions of 0 mM, 10 mM, 15 mM, 20 mM, and 25 mM $Na_2CO_3$ for 7 days (Fig. S1-A). Based on the significant variation of membrane injury (MI), we selected 25 mM $Na_2CO_3$ (pH = 11.2, electrical conductivity (EC) = 3.7 mS $cm^{-1}$) to simulate alkaline stress (Liu et al. 2018). For determining the time point of alkaline stress treatment, the leaves of 25-day-old alfalfa seedlings (approximately the third trifoliate leaf stage) were harvested to determine the MDA content after 1, 3, 5, and 7 days of alkaline treatment. A significant difference was observed among the two cultivars after seven days of alkaline treatment, so we selected the seventh day as the treatment time (Fig. S1-B). For the alkaline stress experiment, the alfalfa cultivar seedlings (GN and AG) were treated with distilled water (the control treatment) and named as CGN and CAG, respectively. The alkaline treatment (25 mM $Na_2CO_3$) groups were named as TGN and TAG, respectively. Then, at the end of each treatment, fully expanded third leaves from the top were collected and stored at -80°C until use in RNA-seq, qRT-PCR analysis, and physiological and biochemical analyses. A randomized block design was used in the experiment, with three replicates for each treatment.

Plant shoot fresh weight was measured immediately after harvesting.

### Determination of membrane injury (MI)

Membrane injury (MI) was measured by electrolyte leakage according to the method of Tantau and Dörffling (1991). Briefly, leaf samples (2 g fresh weight) were submerged in 15 ml of deionized water in 50 ml conical tubes and kept at 20°C for 1 h. The electrical conduction of the effusion was then measured (R1) with a conductivity meter DDS-12 (Lida In., Shanghai, China). The tissue samples were heated in a boiling bath for 40 min, cooled to 20°C, and the electrical conduction of the effusion was measured again (R2). The degree of membrane injury (MI) was evaluated using the formula MI (%) = R1/R2 × 100%.

### Determination of malondialdehyde (MDA) content

The malondialdehyde (MDA) content was determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). The fresh leaf and root samples (0.1 g) were homogenized in 1 ml of 50 mM phosphate buffer (pH 7.8) using a bench-top ball-mill (Scientz-48, Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China) at 50 Hz for 30 s, and then centrifuged at 12,000 rpm for 15 min. Subsequently, 400 ul of supernatant was mixed with 1 ml of 0.5% thiobarbituric acid, and the mixture was placed in a boiling water bath for 20 min. The mixture was then cooled and centrifuged, and the absorbance of the resulting supernatant was measured at 532, 600, and 450 nm. The MDA content was calculated using the following formula: $6.45 * (A_{532} - A_{600}) - 0.56 * A_{450}$.

### Determination of chlorophyll content

The chlorophyll content was determined according to the modified method of Zhang et al. (2017). Leaf samples were extracted using an 8 ml mixture of ethanol and acetone. The absorbance of the supernatant was determined at 645 and 663 nm. Total chlorophyll content was calculated using the following formula: $(8.02\ OD_{663nm} + 20.21\ OD_{645nm})V/1000W$.

### Determination of antioxidant enzyme activities

For the determination of antioxidant enzyme activities, the fresh leaves (0.1 g) were loaded in a 2 ml tube and frozen in liquid nitrogen, then homogenized in 1 ml of 50 mM phosphate buffer (pH 7.8) using a benchtop ball-mill at 50 Hz for 30s. The homogenate was centrifuged at 12,000×*g* for 15 min at 4°C, and the resulting supernatant was used for peroxidase (POD) and catalase (CAT) assays. POD (EC1.11.1.7) activity was determined by assessing the rate of guaiacol oxidation in the presence of $H_2O_2$. One unit of POD was defined as the increase in absorbance per minute at 470 nm (Klapheck et al. 1990). CAT (EC 1.11.1.6) activity was measured according to the method described by Aebi (1984). CAT activity was assayed by the decline in absorbance per minute at 240 nm as a consequence of $H_2O_2$ consumption.

### Determination of soluble sugar and proline contents

The soluble sugar content of each sample was measured following the modified method described by Liu et al. (2009). Approximately 50 mg dry powder sample of the shoot was extracted in 15 mL of double-distilled $H_2O$ and boiled for 20 min. Next, 5 mL of anthrone reagent was added to the 1 mL extract and incubated at 95°C for 20 min, then cooled to room temperature. The solution's absorbance was measured at 620 nm with a spectrophotometer (T6, Puxi General Instrument Co., Ltd., Beijing, China).

The proline content was determined according to the modified method of Bates et al. (1973). Specifically, ca. 50 mg of a dry powder sample from the shoot was homogenized in 5 mL of 3% (w/v) sulfosalicylic acid, and then the mixture was placed in a boiling water bath for 10 min. The 18 mL plastic tubes were cooled and then centrifuged at 3000 ×*g* for 20 min. Approximately 2 mL of the supernatant was mixed with 2 mL of glacial acetic acid and 2 mL of acidic ninhydrin reagent and then boiled for 30 min. The tubes were cooled; 4 mL toluene was added to each tube, and the absorbance at 520 nm was determined with a spectrophotometer (T6, Puxi General Instrument Co. Ltd., Beijing, China).

### Determination of metal ions

Approximately 0.1 g dry powder from each sample was digested with a mixture of $HNO_3$ and $HClO_4$ (v/v = 2:1) and diluted to 100 mL. Using this mixture, the total cation contents of $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ were determined by inductively coupled plasma mass spectrometry (ICPS-7500, Shimadzu Corporation, Japan). The three indices of $K^+/Na^+$ ratio, $Ca^{2+}/Na^+$ ratio, and $Mg^{2+}/Na^+$ ratio in alfalfa shoots were calculated using the corresponding values.

### RNA extraction, cDNA library preparation, and sequencing

Three biological replicates from each cultivar and treatment were used for RNA-seq analysis. Total RNA was isolated from leaf samples using the Trizol reagent (Invitrogen, Thermo Fisher, MA, USA) according to the manufacturer's instructions. RNA purity and integrity were determined with a NanoPhotometer® spectrophotometer (Thermo Fisher, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). A total of 3 μg of RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using a NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). The mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. The cleaved RNA fragments were transcribed into first-strand cDNA using reverse transcriptase, and subsequent second-strand cDNA synthesis was performed using DNA polymerase I and

RNase H. The fragments were ligated to sequencing adaptors. Library preparations were sequenced on an Illumina Novaseq platform, and 150 bp paired-end reads were generated.

### Differentially expressed genes (DEGs) analysis and annotation

Clean reads were mapped to the alfalfa genome using hisat2 (Chen et al. 2020). HTSeq v 0.6 was used to estimate the read count of each gene (Anders et al. 2014). For each transcription region, an FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variation using a custom R script. The differential expression analysis between sample groups was performed using DESeq2 software (Anders and Huber 2010). The genes/transcripts with the parameters of false discovery rate (FDR) below 0.05 and absolute $\log_2$ (fold change) $\geq 1$ were considered significant DEGs. All of the transcripts were subsequently annotated into three public databases, the Non-Redundant Protein Sequence Database (Nr), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

### Quantitative Real-Time PCR (qRT-PCR) analysis

The quantitative Real-Time PCR (qRT-PCR) was performed using an ABI 7300 Real-Time PCR system (Applied Biosystems, USA). The gene-specific PCR primers are listed in Table S1. The qPCR program was as follows: 3 min at 95°C followed by 39 cycles of 95°C for 10 s and 58°C for 30 s. The transcript statistical analysis abundance for each gene was normalized to the internal control gene Actin-2 (GenBank ID: JQ028730.1). Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Each measurement was completed using three biological replicates.

### Statistical analysis

Comparisons among samples were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Variables were presented as means ± standard error, and $P < 0.05$ was considered significant. All of the tests were performed with SPSS Version 20.0 for Windows (SPSS, Chicago. IL, USA).

## Results

### Determination of experimental conditions for alkaline treatment

As depicted in Fig. S1(A), no significant membrane injury (MI) was observed in the leaves of either cultivar under alkaline stress ($Na_2CO_3$) concentrations below 25 mM compared with the control treatment. When the $Na_2CO_3$ concentration was raised to 25 mM, a sharp increase of MI was observed in both cultivars; however, AG showed a 1.39-fold increase of MI compared to GN.

The cultivar AG showed higher MDA content, ranging from 8.77 to 12.29 nmol $g^{-1}$, compared with the range of 6.14–8.50 nmol $g^{-1}$ in GN under alkaline conditions (Fig. S1B). The MDA content of AG after seven days of treatment was significantly higher (12.29 nmol $g^{-1}$) than that of GN (8.50 nmol $g^{-1}$).

Based on these results, we selected 25 mM $Na_2CO_3$ to simulate alkaline stress and seven days for the stress time point in the following experiments.

### Plant growth and physiological responses to alkaline stress

Leaf yellowing was severe in the sensitive cultivar (AG) under the alkaline conditions (Fig. 1A, B). Compared to the control treatment, shoot fresh weight (Fig. 1C) and the chlorophyll content (Fig. 1D) of AG were significantly decreased by 39.17% and 46.69%, respectively, after seven days of alkaline treatment (25 mM $Na_2CO_3$), while GN showed relatively stable growth and chlorophyll content.

Alkaline treatment induced the accumulation of soluble sugar by 1.39-fold and 1.33-fold (Fig. 2A), and proline by 3.46-fold and 2.55-fold in GN and AG, respectively (Fig. 2B). Moreover, alkaline treatment also increased enzyme activities of POD by 4.38-fold and 2.20-fold (Fig. 2C) and CAT by 9.15-fold and 1.86-fold (Fig. 2D) in GN and AG, respectively.

For metal ions, $Na^+$ content (Fig. 3B) was increased, while $K^+$ (Fig. 3A), $Ca^{2+}$ (Fig. 3C) and $Mg^{2+}$ contents (Fig. 3D) and the ratios of $K^+/Na^+$(Fig. 3E), $Ca^{2+}/Na^+$ (Fig. 3F) and $Mg^{2+}/Na^+$ (Fig. 3G) were decreased in both GN and AG in response to alkaline conditions. Nevertheless, compared with AG, GN had relatively lower levels of $Na^+$ (Fig. 3B) but higher levels of $Ca^{2+}$ (Fig. 3C) and $Mg^{2+}$ (Fig. 3D) and the ratios of $Ca^{2+}/Na^+$ (Fig. 3F) and $Mg^{2+}/Na^+$ (Fig. 3G). There was no difference in $K^+$ content (Fig. 3A) or $K^+/Na^+$ ratio (Fig. 3E) between the two cultivars under the alkaline conditions.

### Identification and classification of differentially expressed genes (DEGs)

After the filtration, the reads from RNA-seq were aligned to the alfalfa genome, and the mapped ratio ranged from 76.80 to 78.01% (Table S2). By comparing samples of the two cultivars (GN and AG) under control (CGN, CAG) and alkaline treatment conditions (TGN, TAG), we constructed two comparison groups: TGN vs CGN (622 genes) and TAG vs CAG (541 genes) (Fig. 4A). All DEGs were used for hierarchical cluster analysis of transcription abundance, and the heatmap of DEGs showed that the expression levels of specific and common genes in GN and AG were increased or decreased under alkaline stress (Fig. S2 and Table S3). Subsequently, we classified the DEGs from the above two comparisons into three categories: alkaline-responding genes from the tolerant cultivar GN (TAR), from the sensitive cultivar AG (SAR), and common to both cultivars (CAR, non-cultivar-specific). A Venn diagram of these DEGs indicated that 48 CAR genes responded to alkaline stress (Fig. 4B); 16 genes were co-upregulated, and 20 genes were co-down-regulated in both cultivars (Fig. 4B and Table S4). Five genes were up-regulated in GN but down-regulated in AG (Fig. 4B), and seven genes were down-regulated in GN but up-regulated in AG (Fig. 4B and Table S4). Moreover, 367 and 207 TAR genes (574 in total) were specifically up- and down-regulated in GN, and 266 and 227 SAR genes (493 in total) were specifically up- and down-regulated in AG (Fig. 4B and Table S4).

### GO enrichment analysis for the DEGs in response to alkaline stress

Gene Ontology (GO) analysis was performed to assign functional information to the DEGs. Among the 48 CAR genes, 27 were assigned to 19 GO terms, including five for cellular component (CC), three for molecular function (MF) and 11 for biological process (BP) (Fig. 5A). For the TAR genes, 311 out of 574 were assigned to 37 GO terms, which were summarized under 3 main GO categories, including 12 for CC, 7 for MF, and 18 for BP (Fig. 5B). For the SAR genes, 260 out of 493 were assigned to 33 GO terms, including 10 for CC, 5 for MF, and 18 for BP (Fig. 5C).

The most enriched GO terms in the CC category were cell, cell part, and membrane and organelle for TAR, SAR, and CAR genes, respectively. In the MF category, the main GO terms were catalytic activity and binding and transporter activity for TAR and SAR and catalytic activity, binding, and nucleic acid binding transcription factor activity for CAR genes; in the BP category these were metabolic process, cellular process, and single-organism process in all three DEGs categories (TAR, SAR, and CAR).

### KEGG enrichment analysis for the DEGs in response to alkaline stress

To further investigate the potential functions of DEGs in response to alkaline stress, KEGG enrichment analysis was performed. In this analysis, 11, 110, and 116 DEGs from CAR, TAR, and SAR were classified into 22, 91, and 77 functional categories, respectively (Table S5).

Based on the criteria of a P-value less than 0.05, the three significantly enriched KEGG pathways in CAR were pyrimidine metabolism (two genes), nicotinate and nicotinamide metabolism (one gene), and plant-pathogen interactions (three genes) (Fig. 6A and Table S5). The significantly enriched six KEGG pathways in TAR were biosynthesis of secondary metabolites (47 genes), flavonoid biosynthesis (eight genes), metabolic pathways (55 genes), valine, leucine and isoleucine biosynthesis (three genes), circadian rhythm-plant (five genes) and MAPK signaling pathway-plant (nine genes), respectively (Fig. 6B and Table S5). The significantly enriched six KEGG pathways in SAR were biosynthesis of secondary metabolites (44 genes), glycerophospholipid metabolism (nine genes), metabolic pathways (56 genes), alanine, aspartate and glutamate metabolism (five genes), alpha-Linolenic acid metabolism (four genes) and glycerolipid metabolism (five genes), respectively (Fig. 6C and Table S5).

In total, 27 specific pathways for GN and 13 specific pathways for AG were recognized (Table S5). Of these, pathways involved in valine, leucine and isoleucine biosynthesis (three genes), C5-branched dibasic acid metabolism (two genes), lysine degradation (four genes), pantothenate and CoA biosynthesis (three genes), lysine biosynthesis (two genes) and arginine and proline metabolism

(four genes) were specifically and significantly enriched in GN (P-value < 0.05). However, vitamin B6 metabolism (two genes) was specifically and significantly enriched in AG (P-value < 0.05) (Table S5).

### Identification of core alkaline-responsive genes from CAR category

As shown in Figure 4A, five CAR genes, MS.gene60267.t1, MS.gene021035.t1, MS.gene026390.t1, MS.gene45510.t1 and MS.gene81671.t1, were significantly up-regulated in GN, while being down-regulated in AG (Table 1). Among these, only two genes had pathway annotations: MS.gene021035.t1 encoding 3-ketoacyl-CoA synthase (KCS) was simultaneously mapped into several different pathways, including metabolic pathways, biosynthesis of secondary metabolites, plant-pathogen interaction, and fatty acid elongation; the MS.gene026390.t1 encoding long-chain acyl-CoA synthetase (ACSL) was simultaneously mapped into other pathways, including fatty acid degradation, fatty acid biosynthesis, fatty acid metabolism, and peroxidase metabolism (Table 1). In contrast, seven CAR genes, MS.gene065904.t1, MS.gene65352.t1, MS.gene88914.t1, MS.gene068618.t1, MS.gene031323.t1, MS.gene47982.t1, and MS.gene024840.t1, were significantly down-regulated in GN while being up-regulated in AG (Table 1). Among these, only three genes had pathway annotations: MS.gene024840.t1 encoding 12-oxophytodienoic acid reductase (OPR) is related to metabolic pathway; MS.gene068618.t1 encoding cinnamyl-alcohol dehydrogenase (CAD) is related to phenylpropanoid biosynthesis, and MS.gene031323.t1 encoding replication factor A1 (RFA1) is simultaneously related to DNA replication and repair pathways, including mismatch repair, DNA replication, nucleotide excision repair, and homologous recombination (Table 1).

### Identification of core alkaline-responsive genes from TAR and SAR categories

As GN had higher tolerance to alkaline stress than AG (Wei et al. 2020; Fig. 1), we hypothesized that the DEGs mapped to significant enrichment categories or specific enrichment in GN may contribute to the alkaline tolerance phenotype of alfalfa.

In this study, a total of nine genes from TAR were significantly enriched in MAPK signaling pathway-plant (Table 2). One was an abscisic acid receptor (PYL) (MS.gene32980.t1); one was a pathogenesis-related protein 1 (PR1) (MS.gene02249.t1), and one was a transcription factor MYC2 (MYC2) (MS.gene060503.t1); each was significantly up-regulated in GN (Table 2). Conversely, three genes encoding calmodulin (CALM) (MS.gene070637.t1, MS.gene073306.t1, MS.gene86455.t1), a WRKY transcription factor 33 (WRKY33) (MS.gene24960.t1), an ethylene receptor (ETR) (MS.gene025978.t1) and an ethylene biosynthesis enzyme, 1-aminocyclopropane-1-carboxylate synthase 1/2/6 (ACS 1_2_6) (MS.gene023978.t1), were significantly down-regulated in GN (Table 2).

Compared with TAR, only four genes from SAR were mapped into MAPK signaling pathway-plant. There were two for LRR receptor-like serine/threonine-protein kinase FLS2 (FLS2) (MS.gene002602.t1, MS.gene002602.t1), one for ethylene-responsive transcription factor 1 (ERF1) (MS.gene49293.t1), and one for MAP kinase substrate 1 (MKS1) (MS.gene02674.t1) that were consistently down-regulated in AG under alkaline conditions (Table 2).

Moreover, a total of eight TAR genes were significantly enriched in flavonoid biosynthesis (Table 3). Two genes for chalcone synthase (CHS) (MS.gene024293.t1, MS.gene09285.t1) involved in flavonoid biosynthesis were significantly up-regulated, while another three CHS genes (MS.gene050883.t1, MS.gene88887.t1, MS.gene88888.t1) were significantly down-regulated in GN (Table 3). Notably, the above five CHS genes were also categorized in circadian rhythm-plant (Table 4). In addition, one gene for flavonol synthase (FLS) (MS.gene053758.t1) and two for flavonoid 3'-monooxygenase (CYP75B1) (MS.gene044441.t1, MS.gene79428.t1) were significantly up-regulated in GN (Table 3).

Three SAR genes were significantly enriched in flavonoid biosynthesis: one for anthocyanidin synthase (ANS) (MS.gene20359.t1) and one for bifunctional dihydro flavonol 4-reductase/flavanone 4-reductase (DFR) (MS.gene27250.t1) were significantly up-regulated, while the third gene encoding shikimate O-hydroxycinnamoyl transferase (HCT) (MS.gene90374.t1) involved in flavonoid biosynthesis was significantly down-regulated, in AG (Table 3). In addition, one gene encoding GIGANTEA (GI) (MS.gene88890.t1) involved in plant circadian rhythms was significantly down-regulated in AG (Table 4).

In addition, among the TAR genes, two genes for acetolactate synthase (ALS) (MS.gene06222.t1 and MS.gene38605.t1) and one for dihydroxy acid dehydratase (DHAD) (MS.gene35759.t1) were recognized and mapped into valine, leucine, and isoleucine biosynthesis (Table 5) and were specifically up-regulated in GN. Two SAR genes for pyridoxal phosphate phosphatase (PHOSPHO2) (MS.gene069913.t1 and MS.gene072128.t1) involved in vitamin B6 metabolism were up-regulated in AG (Table 6).

### Verification of gene expression by quantitative real-time PCR (qRT-PCR)

To confirm the reliability of our transcriptome data, we randomly selected eight DEGs, six CAR, one TAR, and one SAR and verified their expression levels by qRT-PCR. Based on the $\log_2$ (fold change) of eight genes, the RNA-seq generated data showed significant linear correlation with the qRT-PCR results (GN, $R^2$= 0.94; AG, $R^2$=0.89) (Fig. S3 and Table S6), suggesting that the expression of these selected genes in our transcriptome data was evidently in good accordance with the qRT-PCR results.

## Discussion

Saline-alkaline (SA) stress, characterized by high $Na^+$, high osmotic shock, and high soil pH, represents a significant constraint to plant growth and productivity. Therefore, improving SA tolerance of plants is an essential way for effective and sustainable utilization of SA-affected lands. Alfalfa is a promising candidate due to its high quality, yield, and saline tolerance (An et al. 2016; Long et al. 2019). However, our knowledge regarding the physiological and molecular mechanisms underlying SA response in alfalfa remains limited. Although the genome of alfalfa has been recently published (Chen et al. 2020), extensive analysis based on the above genome information has not yet been reported. In the present study, the comparative analysis of two alfalfa cultivars with different alkaline sensitivities, the alkaline sensitive cultivar AG and the tolerant cultivar GN, revealed that ROS-scavenging activity, osmolyte accumulation, and ion homeostasis are important physiological mechanisms for SA tolerance. Moreover, transcriptomic analysis further suggested that the acquisition of SA tolerance is accompanied by a wide transcriptional reprograming of genes involved in multiple pathways, including biosynthesis of phenylpropanoids, flavonoids, and some amino acids as well as lipid metabolism and the MAPK signaling pathway. These results provide novel insights into the physiological and genetic mechanisms of alkaline tolerance in alfalfa.

### Physiological regulation in response to alkaline stress

Alkaline stress not only inhibits the seed germination of alfalfa but also seriously affects the growth and development of seedlings (An et al. 2016, Liu et al. 2018, Long et al. 2019). Our previous study also confirmed that the alkaline tolerance of alfalfa was associated with the cultivar's genetic characteristics (Wei et al. 2020). As shown by our results, AG is more sensitive to alkaline stress than GN, as reflected by the decreased shoot fresh weight and leaf chlorophyll contents (Fig. 1C, D). Similar results were reported in the alkaline-stressed halophyte *Puccinellia tenuiflora* (Yin et al. 2019), suggesting that alkaline stress has adverse effects on photosynthesis and inhibits the normal growth of alkaline-sensitive cultivars.

MDA reflecting the degree of membrane lipid peroxide is usually used to indicate the degree of oxidative stress (Del Rio et al. 2005). Alkaline stress can cause excessive accumulation of ROS in plants, leading to cell damage and even death through oxidative stress (Choudhury et al. 2017; Sewelam et al. 2016). Meanwhile, alkaline stress can also induce increases in antioxidant activity (Sewelam et al. 2016; Choudhury et al. 2017; Zhang et al. 2017; Liu et al. 2019). In the present study, the MDA contents in GN were significantly lower than in AG (Fig. S1-B), and GN promoted POD and CAT activities compared with AG (Fig. 2C, D), indicating that alfalfa can maintain a balance of the generation and elimination of ROS by increasing the activities of these antioxidant enzymes under alkaline conditions.

High salt concentrations leads to loss of K and Ca due to the depolarization of membranes and the displacement by Na ions (Cramer et al. 2006). The presence of a large amount of $Na^+$ in alkaline solution disturbs the ionic homeostasis in plants, and triggers a higher pH environment in the rhizosphere, thus causing the precipitation of $Ca^{2+}$ and $Mg^{2+}$ ($Ca_2CO_3$ and $Mg_2CO_3$), further resulting in nutrient imbalance (Wang et al. 2012). Our data showed that the contents of $K^+$, $Ca^{2+}$ and $Mg^{2+}$ in the shoots of GN and AG were significantly lower under alkaline stress compared with the favorable conditions (Fig. 3). Interestingly, GN maintained higher accumulation of $Ca^{2+}$and $Mg^{2+}$, and the ratios of these two ions to $Na^+$ in shoots were also higher than those in AG, suggesting that accumulation profiles of $Ca^{2+}$and $Mg^{2+}$ may play an important role in maintaining ionic homeostasis in alfalfa under alkaline stress (Wei et al. 2020). Based on the above results, we speculate that the transient increase in $Ca^{2+}$ levels of alkaline-treated plants might activate alkaline signaling pathways in the plant and be involved in regulating anti-stress genes expressed in response to alkaline stress.

Proline and soluble sugar participate in the physiological response of plants by osmotic regulation (Majid 2012; Rokebul et al. 2017). In the present study, GN accumulated more soluble sugar and proline than in AG under alkaline stress (Fig. 2A, B). This is

consistent with previous studies (Li et al. 2010; Rokebul et al., 2017; Sairam, Rao et al. 2002) suggesting that overaccumulation of proline can significantly improve the plant tolerance to alkaline stress. However, alkaline stress induced slightly higher soluble sugar in GN than in AG. This might be attributed to the cultivar differences or to alkaline stress intensity leading to changes in sugar accumulation (Ashrafi et al. 2014).

### Core alkaline-responsive genes related to cell well and membrane protection

As reported previously, the monomers of lignin are synthesized through the phenylpropanoid pathway (Whetten and Sederoff 1995), while CAD is a key enzyme for lignin biosynthesis (Yang et al. 2018). Lignin provides mechanical strength for plant secondary cell walls, not only protecting the cells from abiotic stress but also serving as the structures in response to environmental stress (Yang and Guo 2017). Lignin downregulation can lead to constitutive defense response expression in alfalfa (Gallego-Giraldo et al. 2011). Consistent with this, our study indicated that one CAR gene encoding CAD (MS.gene068618.t1) involved in phenylpropanoid biosynthesis was down-regulated in GN but up-regulated in AG (Table 1), demonstrating that cell wall damage and altered components in response to alkaline stress are possibly alkaline-sensing mechanisms that would further trigger plant alkaline responses.

Fatty acids, as components of molecules or acting individually, have diverse functions in cell membranes in energy metabolism and in signaling (De Carvalho and Caramujo 2018). They also contribute to inducible stress resistance through the remodeling of the membrane fluidity and defending against the accumulation or excess production of ROS (Halliwell 2006; Iba 2002). Our transcriptome data showed that two CAR genes, ACSL (MS.gene026390.t1) and KCS (MS.gene021035.t1), which are involved in fatty acid elongation, fatty acid degradation, fatty acid biosynthesis, and fatty acid metabolism, were significantly up-regulated in GN while being down-regulated in AG (Table 1). This is in line with the salt tolerance of sorghum and neutral salt or alkaline salt tolerance of rice (Ahmad et al. 2015; Yang et al. 2018). Consequently, these results illustrate that GN can enhance cell membrane protection by up-regulating genes involved in lipid metabolism.

### Core alkaline-responsive genes related to DNA replication and repair

Cells respond to DNA damage by activating a robust DNA damage response (DDR) pathway that provides enough time for specific DNA repair to physically remove the damage in a substrate-dependent manner. To date, five DDR pathways (base combination repair (BER), nucleotide recombination repair (NER), mismatch repair (MMR), homologous recombination repair (HR), and non-homologous end-joining (NHEJ) have been reported (Chatterjee and Walker 2017; Nisa et al. 2019). It has been demonstrated that salinity stress affects the cellular processes involved in DNA replication (Geng et al. 2020; John et al. 2016; Sanan-Mishra et al. 2005). Our study observed that the CAR gene (MS.gene031323.t1) required for DNA replication, mismatch repair, nucleotide excision repair and homologous recombination was down-regulated in GN but significantly up-regulated in AG (Table 1). However, this is inconsistent with the finding that the DNA replication pathway is specific in response to high neutral salt stress rather than high saline-alkaline levels in sugar beets (Geng, Li et al. 2020). We found that the genes involved in DNA replication and repair pathways were differentially expressed whether in tolerant or sensitive alfalfa.

### Core alkaline-responsive genes related to MAPK signaling pathway-plant

The MAPK signaling pathway plays critical roles in diverse processes, including plant growth, development, and response to abiotic stress (Colcombet and Hirt 2008). MAPK cascades are involved in pathogen signaling (Meng and Zhang 2013), and the three TAR genes encoding WRKY33 (MS.gene24960.t1), ACS1_2_6 (MS.gene023978.t1) and PR1 (MS.gene02249.t1) involved in pathogen signaling were significantly differentially expressed in GN (Table 2). However, the FLS2 gene (MS.gene002602.t1 MS.gene36471.t1) and the MKS1 gene (MS.gene02674.t1) involved in pathogen signaling were significantly down-regulated in AG. Additionally, MAPK cascades are also modulated by stress-related phytohormones (Jagodzik et al. 2018). In this study, the TAR gene for ETR (MS.gene025978.t1) and the SAR gene for ERF1 (MS.gene49293.t1) involved in ethylene biosynthesis were uniformly down-regulated in the two cultivars. The TAR gene encoding MYC2 (MS.gene060503.t1) involved in JA biosynthesis and the TAR gene encoding PYL (MS.gene32980.t1) involved in ABA biosynthesis was significantly up-regulated in GN (Table 2). Takahashi's research suggested that MPK8 cascades may mediate $Ca^{2+}$ and ROS signaling in early wound response to prevent the cells from over accumulation of ROS, thus ensuring or retaining an appropriate concentration of ROS as signaling molecules (Takahashi et al. 2011). Consistently, three genes encoding CaLM (MS.gene070637.t1, MS.gene073306.t1, and MS.gene86455.t1) involved in MAPK

signaling pathway-plant were down-regulated in GN (Table 2), indicating that GN possibly enhances alkaline stress tolerance via the MAPK signaling pathway.

### Core alkaline responsive genes related to ROS homeostasis

Flavonoids are a large family of plant secondary metabolites associated with intracellular ROS elimination, and as signal molecules they also interact with transcription factors, kinases, and histones to regulate gene expression at different levels (Ithal and Reddy 2004; Pourcel et al. 2007). Typical enzymes of FLS, CHS, CYP75B1, ANS, and DFR related to the flavonoid biosynthesis were identified. Recent investigations revealed that both saline and saline-alkaline stresses induced flavonoid accumulation (An et al. 2016; Jia et al. 2019; Winkel-Shirley 2002). As expected, we found that eight TAR genes, five genes for CHS (MS.gene024293.t1, MS.gene09285.t1, MS.gene050883.t1, MS.gene88887.t1, MS.gene88888.t1), one FLS gene (MS.gene053758.t1), and two CYP75B1 gene (MS.gene044441.t1, MS.gene79428.t1) involved in flavonoid biosynthesis were differentially induced in GN. Nevertheless, the three SAR genes, encoding ANS (MS.gene20359.t1), HCT (MS.gene90374.t1) and DFR (MS.gene27250.t1) involved in flavonoid biosynthesis were also differentially induced in AG (Table 3). Overall, the induced expression of genes participating in flavonoid metabolism may contribute to enhancing ROS detoxification under alkaline stress.

### Core alkaline-responsive genes related to plant circadian rhythms

As reported previously, CHS is not only a key enzyme for flavonoid synthesis but also is controlled by a distinct circadian clock, and CHS plays an important role in protecting plants from ultraviolet radiation and pathogens (Thain et al. 2002). Furthermore, these unique plant compounds play a major role in enhancing the susceptibility of plants to environmental stresses (Takeuchi et al. 2014; Thain et al. 2002). In the present study, the induced up- or down-regulated TAR genes encoding CHS were also mapped to the category circadian rhythm–plant (Table 4). In addition, GIGANTEA (GI) was originally identified based on its activity in photoperiodic flowering and the circadian clock (Martin-Tryon et al. 2007). GI can also activate the SOS1 and export $Na^+$ out of the cell to achieve salt tolerance (Kim et al. 2013; Park et al. 2016). Our data showed that the SAR gene encoding GI and involved in plant circadian rhythms was significantly down-regulated. However, few reports have focused on the relationship between GI and abiotic stress besides reports for *Arabidopsis* and *Populus* (Kim et al. 2013; Ke et al. 2017). These findings imply that alfalfa may initiate the expression of some genes involved in plant circadian rhythms in response to alkaline stress, but the reason for these gene expression changes remains elusive.

### Core alkaline-responsive genes related to other cultivar-specific pathways

Valine (Val), leucine (Leu), and isoleucine (Ile) are grouped as branched-chain amino acids (BCAAs). Current evidence suggests that both ALS and DHAD are important proteins, and that they function in catalyzing the first step in the synthesis of BCAAs. Previous study has confirmed that the BCAA homeostasis contributes to the adaptation of *Arabidopsis* and *Poplar* to salinity stress (Wang et al. 2020; Zhang et al. 2015). In this study, two TAR genes, an ALS gene (MS.gene06222.t1, MS.gene38605.t1), and a DHAD gene (MS.gene35759.t1), were identified to be specifically involved in valine, leucine, and isoleucine biosynthesis and were up-regulated by alkaline stress (Table 5). Therefore, we speculate that these genes might play critical roles in the regulation of alkaline tolerance in the tolerant cultivar GN by affecting the biosynthesis of valine, leucine, and isoleucine.

Vitamin B6 is an essential co-factor for branched-chain amino acid transaminase (BCAT), the last step of BCAA synthesis (Amorim et al. 2016). Exogenous addition of vitamin B6 can improve the nutritional quality and stress tolerance of crop plants. Furthermore, vitamin B6 has also been shown to function as a potent antioxidant molecule (Vanderschuren et al. 2013). Numerous studies have revealed that plants with lower vitamin B6 are more sensitive to high concentrations of salt, alkali, sucrose, and mannose, as well as oxidative stress (Chen and Xiong 2005; Titiz et al. 2006; Zou et al. 2020). Consistent with these reports, our data showed that two SAR genes, MS.gene069913.t1 and MS.gene072128.t1 encoding PHOSPHO2, were significantly up-regulated in response to the alkaline stress (Table 6). The results suggested that the sensitivity of AG to the alkaline stress was related to the $DEG_S$ involved in vitamin B6 metabolism.

## Conclusions

The cultivar GN exhibited relatively high tolerance to alkaline stress compared with AG, as shown by the higher survival rate and greater shoot biomass under alkaline conditions. Our comparative analysis using these two cultivars suggested that the alkaline tolerance observed in GN is associated with activation of multiple mechanisms in response to alkaline stress, including homeostasis of redox and ion status, accumulation of osmolytes, and activation of diverse metabolic and signaling pathways. Further studies of these pathways and the related potential core genes will contribute to better understanding of the genetic basis for alkaline tolerance, and thereby to the improvement of alfalfa cultivars for increased and sustainable production in SA-affected land.

## Data Availability

The raw RNA-Seq data was available at NCBI-SRA (PRJNA692570) in fastq format.
https://www.ncbi.nlm.nih.gov/bioproject/PRJNA692570

## Declarations


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### Author contributions

Chang-Jie Jiang and Zheng-Wei Liang conceived and designed the experiments; Tian-Jiao Wei, Guang Li, Ming-Ming Wang, Yang-Yang Jin, Guo-Hui Zhang, Miao Liu and Hao-Yu Yang participated in the experiments; Tian-Jiao Wei and Guang Li analyzed the data and wrote the preliminary manuscript; Chang-Jie Jiang revised the manuscript. All authors have read and approved the final manuscript.

### Compliance with ethical standards

**Conflicts of interest** The authors declare no conflict of interest.

## Abbreviations

DEG, Differentially expressed gene; NCBI, National Center for Biotechnology Information; FPKM, Fragments per kilobase per million fragments mapped; GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; MDA, Malonaldehyde; MI, Membrane injury; Nr, Non-redundant protein sequence; POD, Peroxidase; CAT, Catalase; ROS, Reactive oxygen specie; qRT-PCR, quantitative real-time PCR; ACSL, long-chain acyl-CoA synthetase; KCS, 3-ketoacyl-CoA synthase; OPR, 12-oxophytodienoic acid reductase; ALS, acetolactate synthase; DHAD, dihydroxyacid dehydratase; PHOSPHO2, pyridoxal phosphate phosphatase; CAD, cinnamyl-alcohol dehydrogenase; RFA1, replication factor A1; BCAAs, branched-chain amino acids.

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# Tables

**Table 1** Core alkaline-responsive genes from common (non-cultivar-specific) alkaline-responsive genes (CAR)

| | | GN | | AG | | |
|---|---|---|---|---|---|---|
| Gene ID | Desc | FDR | $Log_2$ (fold-change) | FDR | $Log_2$ (fold-change) | KEGG |
| MS.gene021035.t1 | KCS; 3-ketoacyl-CoA synthase | 0.029803024 | 1.836939177 | 0.034994871 | -1.788797598 | Metabolic pathways<br>Biosynthesis of secondary metabolites<br>Plant-pathogen interaction<br>Fatty acid elongation |
| MS.gene026390.t1 | ACSL, fadD; long-chain acyl-CoA synthetase | 0.003432364 | 1.280394653 | 0.002540497 | -1.293164499 | Fatty acid degradation<br>Fatty acid biosynthesis<br>Fatty acid metabolism<br>Peroxidase metabolism |
| MS.gene068618.t1 | CAD; cinnamyl-alcohol dehydrogenase | 0.021771125 | -3.531260951 | 0.038718799 | 3.169581064 | Phenylpropanoid biosynthesis<br>Metabolic pathways |
| MS.gene031323.t1 | RFA1, replication factor A1 | 0.004642951 | -5.627682166 | 0.018263777 | 3.271642887 | Mismatch repair<br>DNA replication, nucleotide excision repair and homologous recombination |
| MS.gene024840.t1 | OPR; 12-oxophytodienoic acid reductase | 0.000453705 | -4.488825706 | 0.000999181 | 3.785840503 | Metabolic pathways |
| MS.gene60267.t1 | | 0.002716409 | 2.705174949 | 0.030251913 | -2.110291933 | |
| MS.gene45510.t1 | | 0.001879191 | 1.610639104 | 0.02814976 | -1.220709706 | |
| MS.gene81671.t1 | | 0.000419616 | 1.342662567 | 0.004761358 | -1.109818235 | |
| MS.gene065904.t1 | | 0.018884337 | -1.311414326 | 0.0014752 | 1.614307527 | |
| MS.gene65352.t1 | | 0.048097353 | -1.772792565 | 0.024717949 | 1.929866472 | |
| MS.gene88914.t1 | | -2.239916034 | -2.239916034 | 0.014354965 | 2.265427795 | |
| MS.gene47982.t1 | | 0.013513536 | -4.443705299 | 0.015995317 | 3.528549555 | |

Legend: “FDR” means false discovery rate; “-“ means the expression of the DEG was unchanged under alkaline stress

**Table 2** Core alkaline-responsive genes related to MAPK signaling pathway-plant

| | | GN | | AG | |
|---|---|---|---|---|---|
| Gene ID | Desc | FDR | $Log_2$ (fold-change) | FDR | $Log_2$ (fold-change) |
| MS.gene070637.t1 | CALM; calmodulin | 0.000118323 | -2.227705926 | - | - |
| MS.gene073306.t1 | CALM; calmodulin | 0.005399313 | -2.252739089 | - | - |
| MS.gene86455.t1 | CALM; calmodulin | 0.000000594 | -2.40757207 | - | - |
| MS.gene24960.t1 | WRKY33; WRKY transcription factor 33 | 0.025706102 | -1.681050062 | - | - |
| MS.gene025978.t1 | ETR, ERS; ethylene receptor | 0.000265193 | -1.218422056 | - | - |
| MS.gene023978.t1 | ACS1_2_6; 1-aminocyclopropane-1-carboxylate synthase 1/2/6 | 0.004294971 | -7.120522911 | - | - |
| MS.gene060503.t1 | MYC2; transcription factor MYC2 | 0.016373805 | 1.677568493 | - | - |
| MS.gene02249.t1 | PR1; pathogenesis-related protein 1 | 0.039314913 | 1.871000829 | - | - |
| MS.gene32980.t1 | PYL; abscisic acid receptor PYR/PYL family | 0.010893986 | 1.977308702 | - | - |
| MS.gene002602.t1 | FLS2; LRR receptor-like serine/threonine-protein kinase FLS2 | - | - | 0.0000041 | -1.041953061 |
| MS.gene36471.t1 | FLS2; LRR receptor-like serine/threonine-protein kinase FLS2 | - | - | 0.000309847 | -2.431812176 |
| MS.gene49293.t1 | ERF1; ethylene-responsive transcription factor 1 | - | - | 0.004644332 | -3.577673396 |
| MS.gene02674.t1 | MKS1; MAP kinase substrate 1 | - | - | 0.015469827 | -1.131531663 |

Legend: “FDR” means false discovery rate; “-“ means the expression of the DEG was unchanged under alkaline stress

**Table 3** Core alkaline-responsive genes related to flavonoid biosynthesis

| | | GN | | AG | |
|---|---|---|---|---|---|
| Gene ID | Desc | FDR | $Log_2$ (fold-change) | FDR | $Log_2$ (fold-change) |
| MS.gene024293.t1 | CHS; chalcone synthase | 0.000199443 | 4.165239646 | - | - |
| MS.gene09285.t1 | CHS; chalcone synthase | 0.029120725 | 2.335911243 | - | - |
| MS.gene050883.t1 | CHS; chalcone synthase | 0.00154111 | -3.438902315 | - | - |
| MS.gene88887.t1 | CHS; chalcone synthase | 0.020171865 | -3.583163827 | - | - |
| MS.gene88888.t1 | CHS; chalcone synthase | 0.029636413 | -2.560289548 | - | - |
| MS.gene053758.t1 | FLS; flavonol synthase | 0.0000315 | 3.815768757 | - | - |
| MS.gene044441.t1 | CYP75B1; flavonoid 3'-monooxygenase | 0.044115664 | 4.601048076 | - | - |
| MS.gene79428.t1 | CYP75B1; flavonoid 3'-monooxygenase | 0.0000184 | 3.734183652 | - | - |
| MS.gene20359.t1 | ANS; anthocyanidin synthase | - | - | 0.038860893 | 5.020415237 |
| MS.gene90374.t1 | HCT; shikimate O-hydroxycinnamoyl transferase | - | - | 0.00108099 | -2.293017237 |
| MS.gene27250.t1 | DFR; bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase | - | - | 0.0000193 | 8.8226616 |

Legend: “FDR” means false discovery rate; “-“ means the expression of the DEG was unchanged under alkaline stress

**Table 4** Core alkaline-responsive genes related to plant circadian rhythms

| | | GN | | AG | |
|---|---|---|---|---|---|
| Gene ID | Desc | FDR | $Log_2$ (fold-change) | FDR | $Log_2$ (fold-change) |
| MS.gene024293.t1 | CHS; chalcone synthase | 0.000199443 | 4.165239646 | - | - |
| MS.gene09285.t1 | CHS; chalcone synthase | 0.029120725 | 2.335911243 | - | - |
| MS.gene050883.t1 | CHS; chalcone synthase | 0.00154111 | -3.438902315 | - | - |
| MS.gene88887.t1 | CHS; chalcone synthase | 0.020171865 | -3.583163827 | - | - |
| MS.gene88888.t1 | CHS; chalcone synthase | 0.029636413 | -2.560289548 | - | - |
| MS.gene88890.t1 | GI; GIGANTEA | - | - | 0.045557002 | -1.655686541 |

Legend: “FDR” means false discovery rate; “-“ means the expression of the DEG was unchanged under alkaline stress

**Table 5** Core alkaline-responsive genes related to valine, leucine, and isoleucine biosynthesis

| | | GN | | AG | |
|---|---|---|---|---|---|
| Gene ID | Desc | FDR | $Log_2$ (fold-change) | FDR | $Log_2$ (fold-change) |
| MS.gene06222.t1 | ALS; Acetolactate synthase I/II/III large subunit | 0.005964661 | 1.265390803 | - | - |
| MS.gene38605.t1 | ALS; Acetolactate synthase I/II/III large subunit | 0.012188049 | 1.681924606 | - | - |
| MS.gene35759.t1 | DHAD; dihydroxy-acid dehydratase | 0.005340854 | 1.057384275 | - | - |

Legend: “FDR” means false discovery rate; “-“ means the expression of the DEG was unchanged under alkaline stress

**Table 6** Core alkaline-responsive genes related to vitamin B6 metabolism

| | | GN | | AG | |
|---|---|---|---|---|---|
| Gene ID | Desc | FDR | $Log_2$ (fold-change) | FDR | $Log_2$ (fold-change) |
| MS.gene069913.t1 | PHOSPHO2; pyridoxal phosphate phosphatase PHOSPHO2 | - | - | 0.01986017 | 1.634540404 |
| MS.gene072128.t1 | PHOSPHO2; pyridoxal phosphate phosphatase PHOSPHO2 | - | - | 0.00483577 | 1.572873832 |

Legend: “FDR” means false discovery rate; “-“ means the expression of the DEG was unchanged under alkaline stress

## Figures

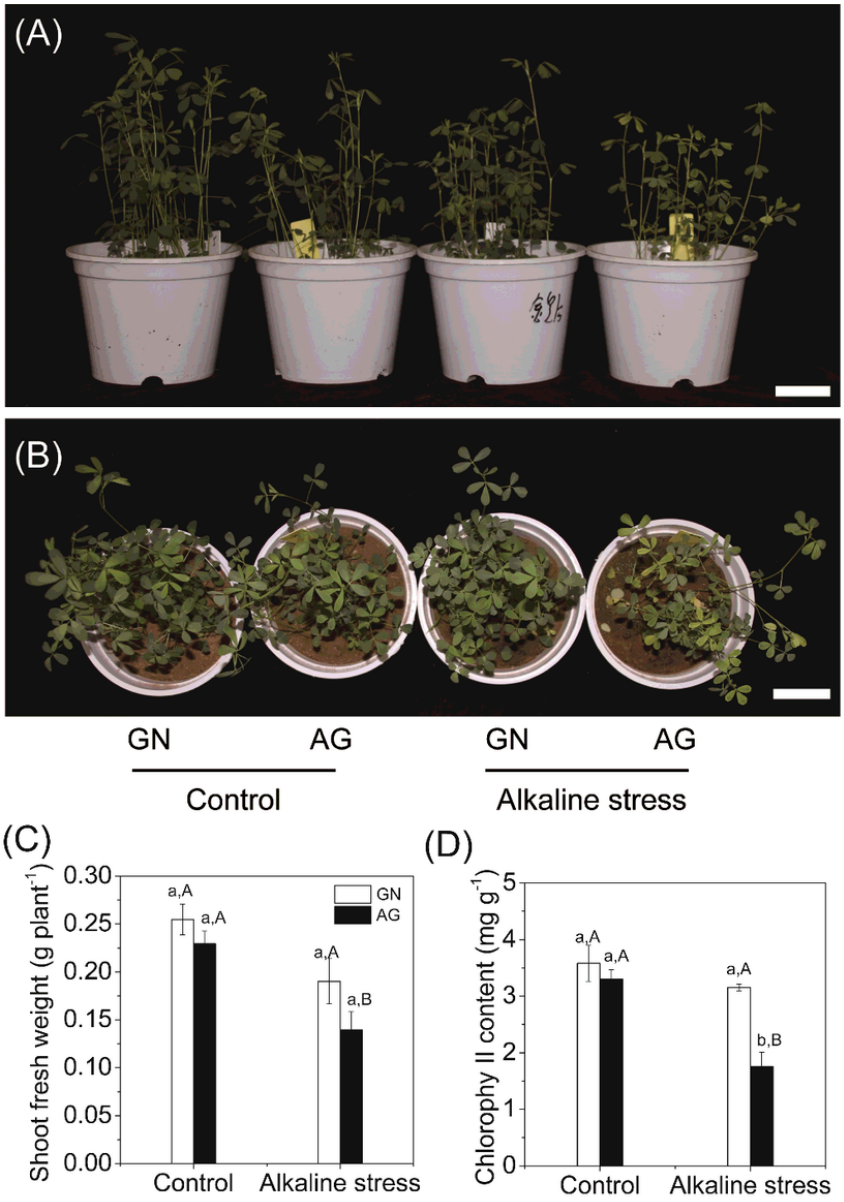



**Figure 1**

Changes in biomass and chlorophyll content of alfalfa seedlings under control or alkaline stress. A-B Photographs of seedling growth were taken after alkaline treatment for 7 days. Bar = 5 cm. C The fresh weight of shoots and D chlorophyll II content in the leaves of seedling were measured after alkaline treatment for 7 days. All values are shown as mean ± standard error. Different lowercase letters (a, b) represent significant differences between cultivars; different uppercase letters (A, B) indicate significant differences between treatments (Tukey's test; $P < 0.05$)

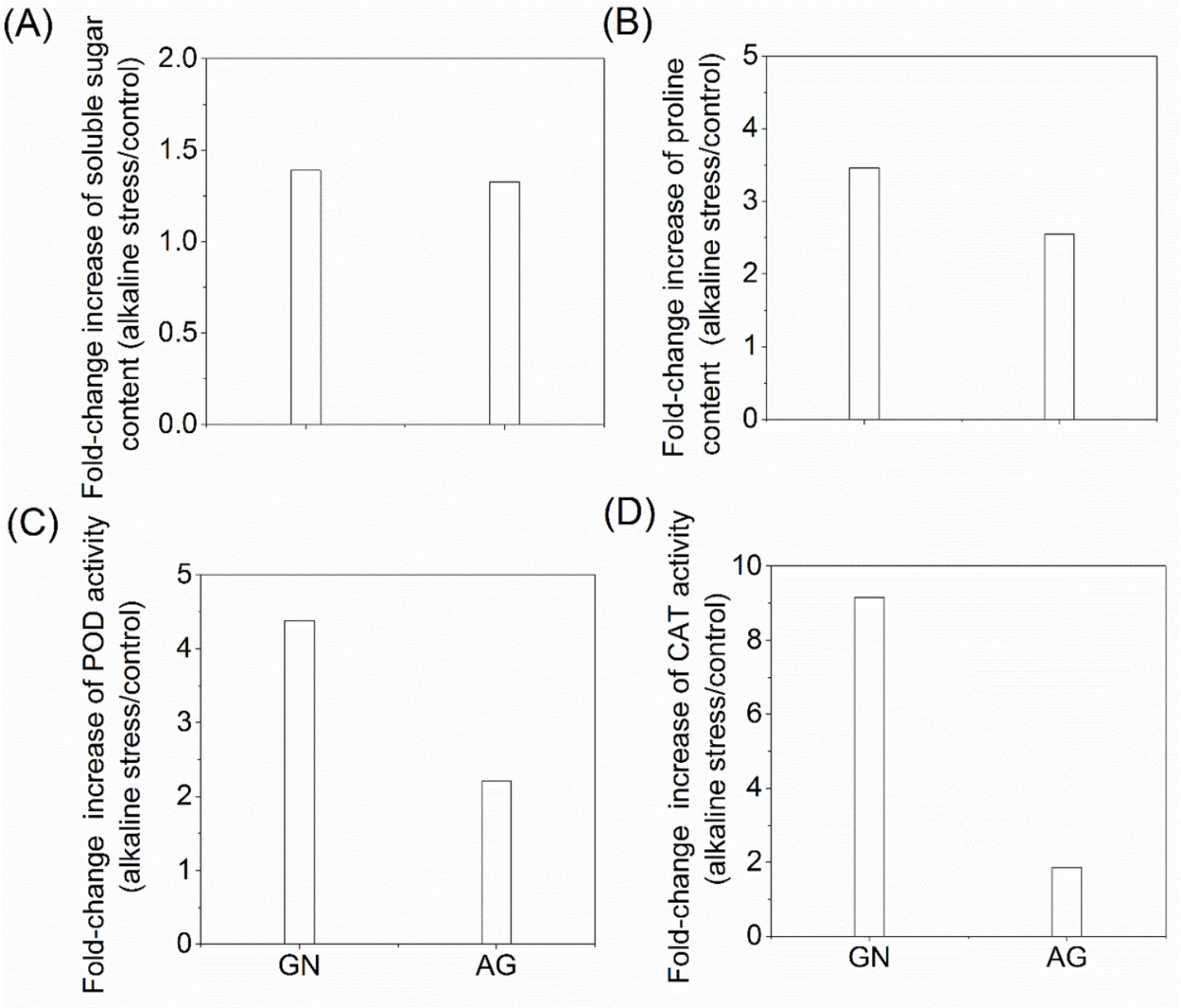
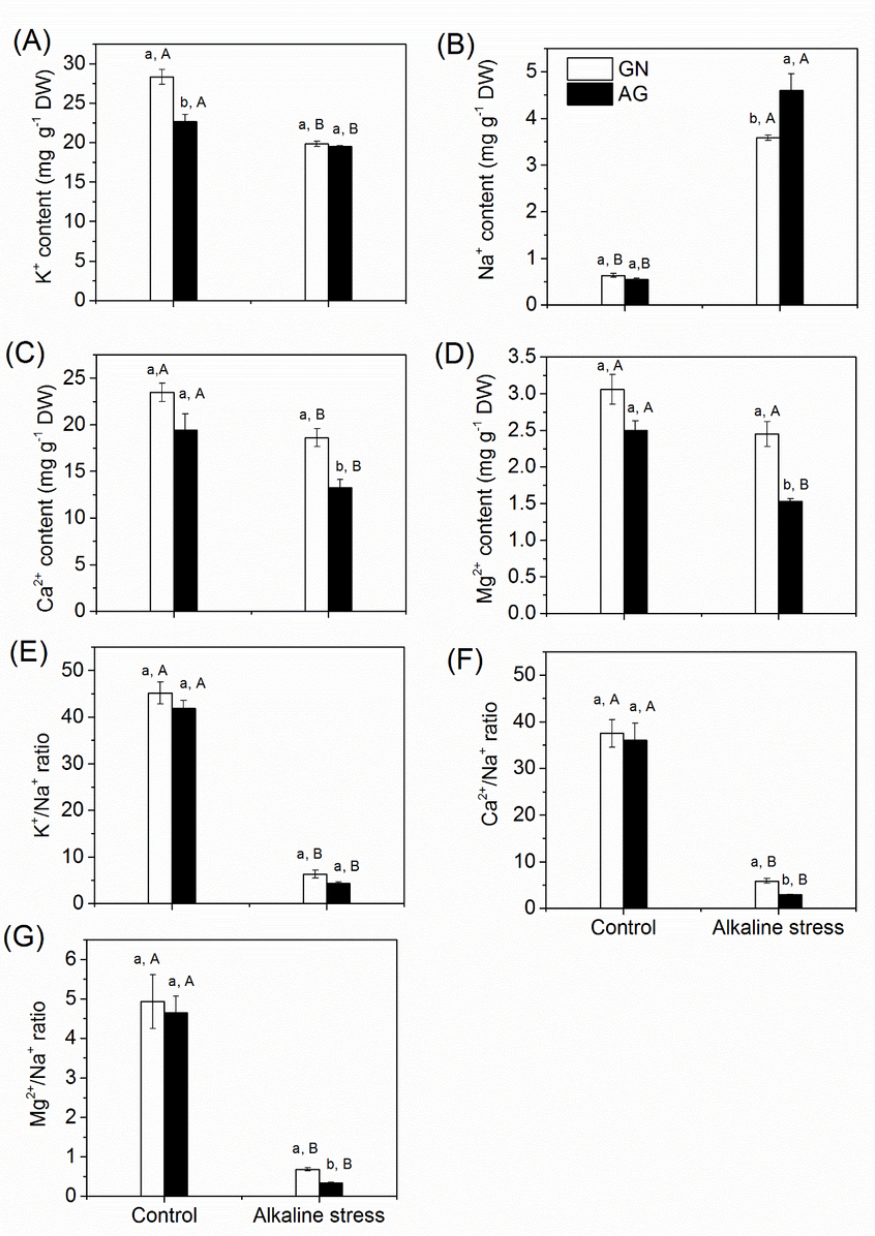



**Figure 2**

Fold-change increases in osmotic regulator content and antioxidant enzyme activities of alfalfa seedlings under alkaline stress. A-B The soluble sugar content and proline content in the shoots of alfalfa seedlings in response to alkaline stress. C-D The activities of peroxidase (POD) and catalase (CAT) in the leaves of alfalfa seedlings in response to alkaline stress. All indices are calculated by taking the control as 1 under alkaline stress conditions (alkaline stress/control)




## Figure 3

Changes in metal ion contents of alfalfa seedlings under control or alkaline stress. A-G The contents of K+, Na+, Ca2+, Mg2+, and their ratios to Na+ in response to alkaline stress. All values are shown as mean ± standard error. Different lowercase letters (a, b) represent significant differences between cultivars; different uppercase letters (A, B) indicate significant differences between treatments (Tukey's test; $P < 0.05$)

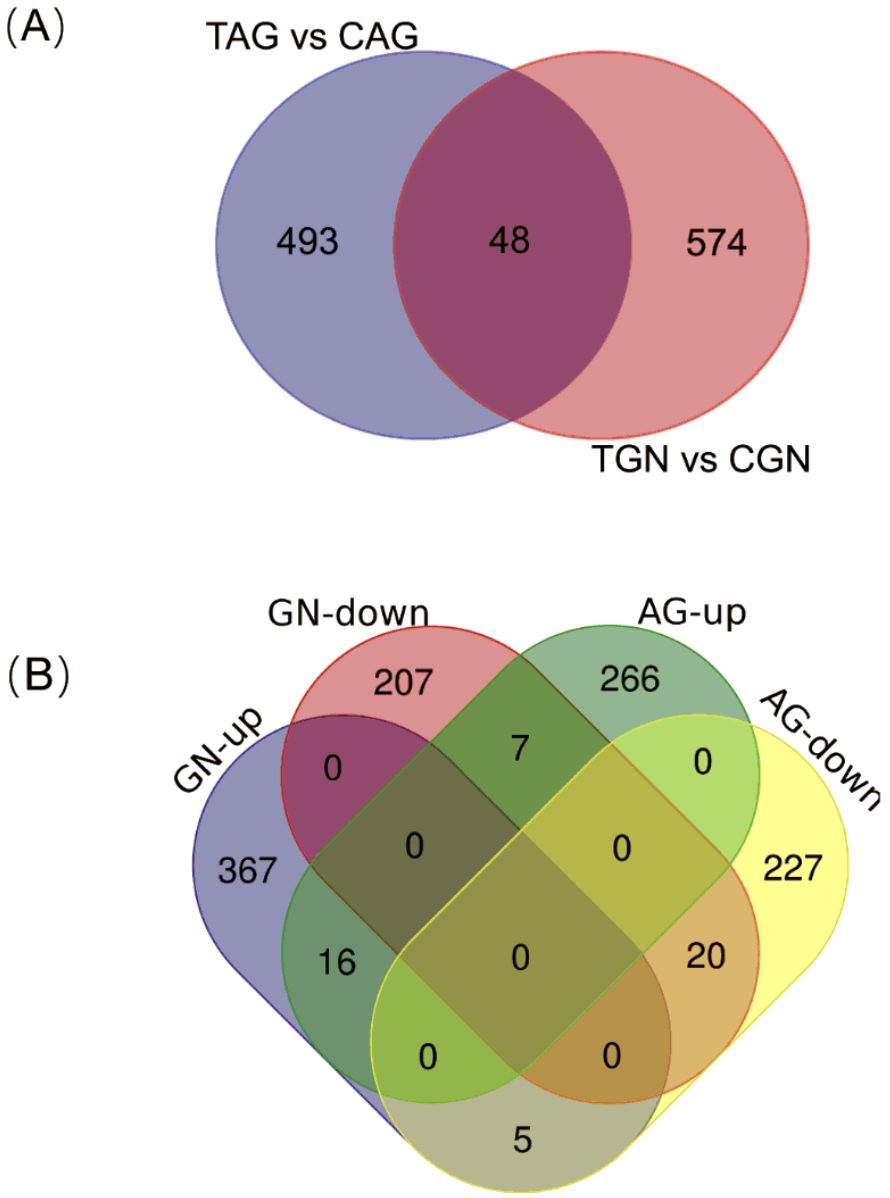
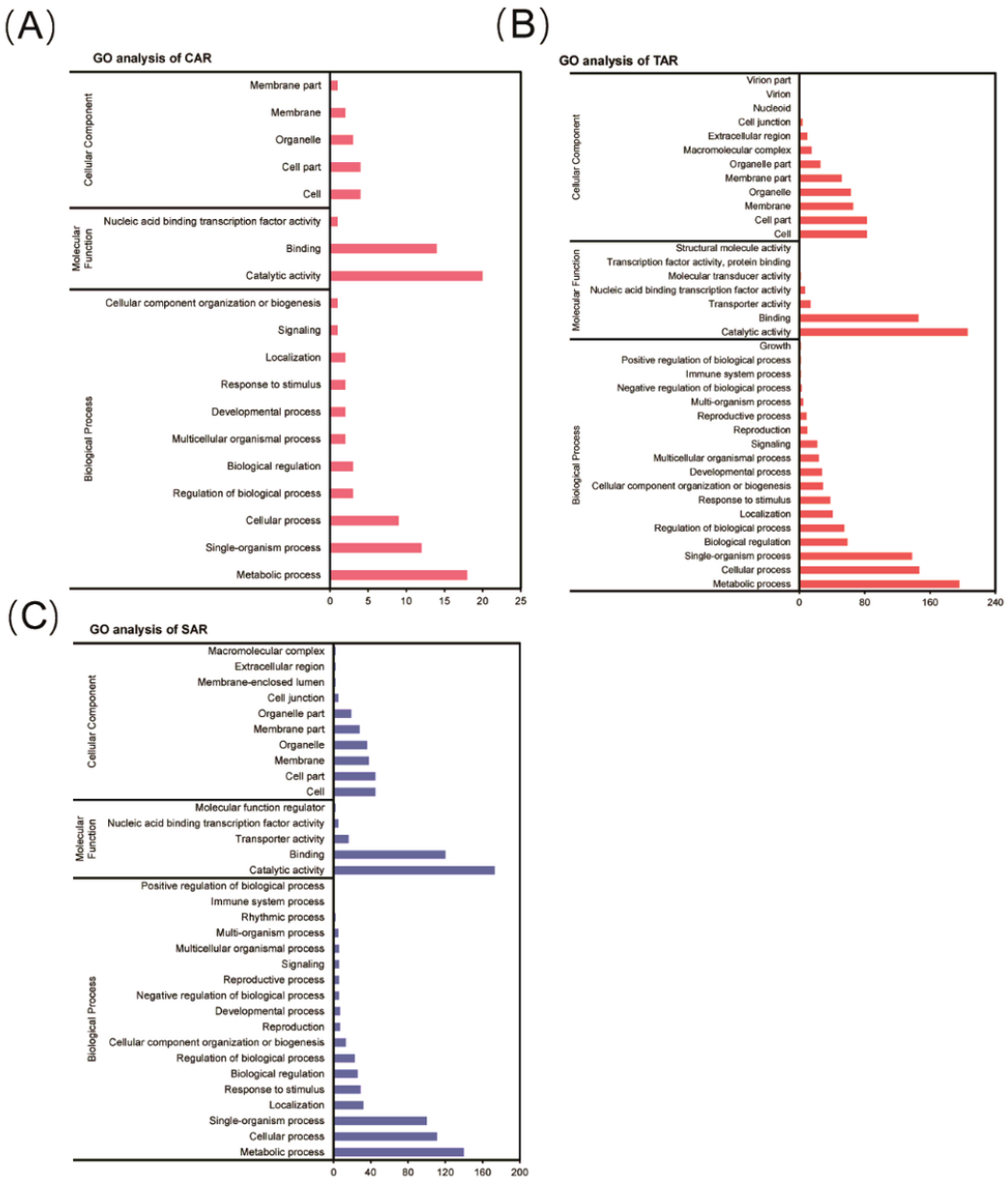


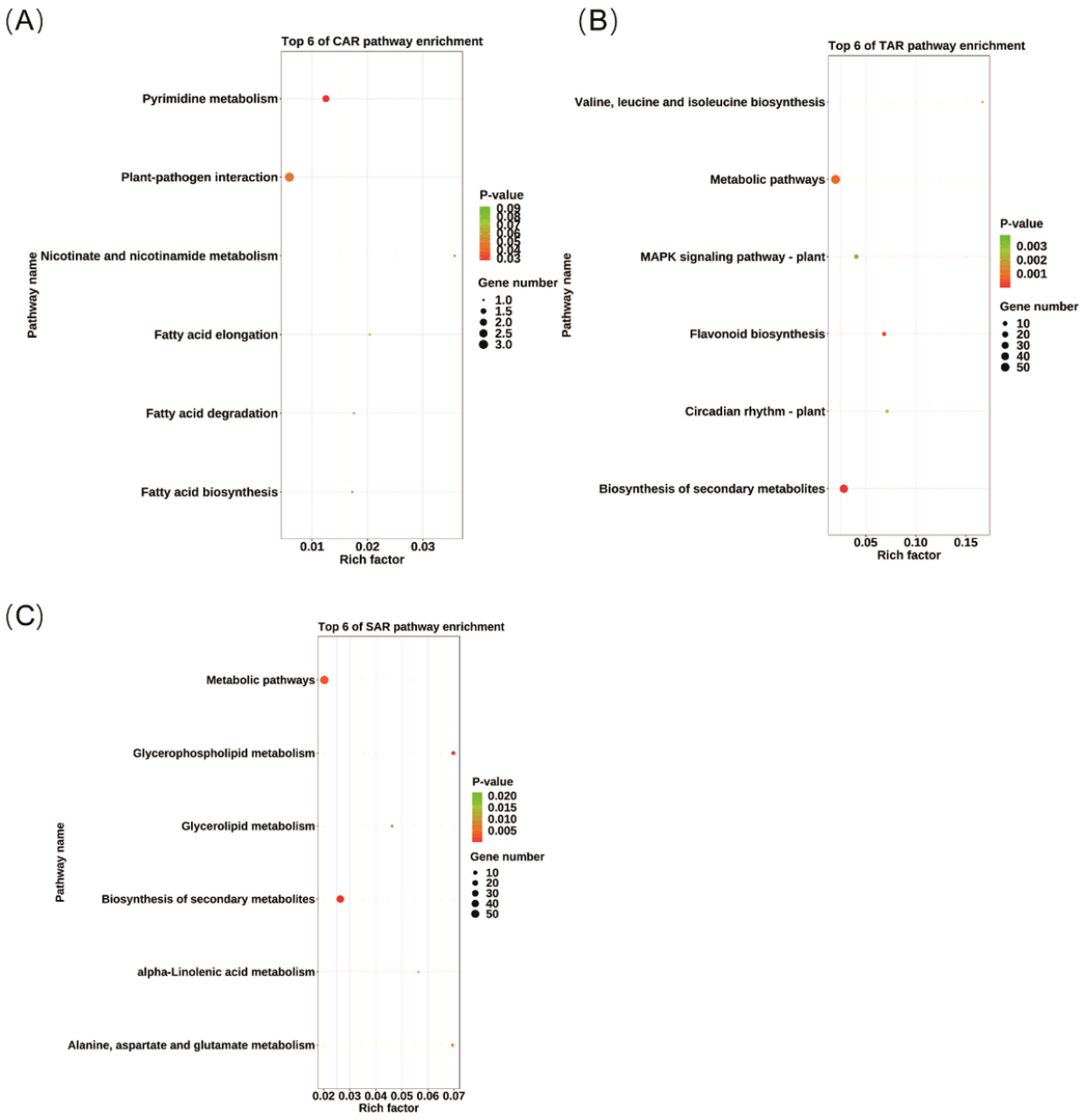

**Figure 4**

Venn diagrams for DEGs of alfalfa in the two comparison groups TGN vs CGN, TAG vs CAG from RNA-seq data. A Summary of the number of DEGs in the presence and absence of alkaline stress. B The Venn diagram shows the up-regulated and down-regulated genes from comparisons between TGN and CGN and between TAG and CAG




## Figure 5

GO analysis for DEGs identified in A CAR, B TAR, and C SAR. CAR represent alkaline-responding genes from the common to both cultivars, TAR represent alkaline-responding genes from the tolerant cultivar GN, and SAR represent alkaline-responding genes from the sensitive cultivar AG




## Figure 6

The 6 most enriched KEGG pathways among the DEGs identified in A CAR, B TAR, and C SAR. CAR represent alkaline-responding genes from the common to both cultivars, TAR represent alkaline-responding genes from the tolerant cultivar GN, and SAR represent alkaline-responding genes from the sensitive cultivar AG

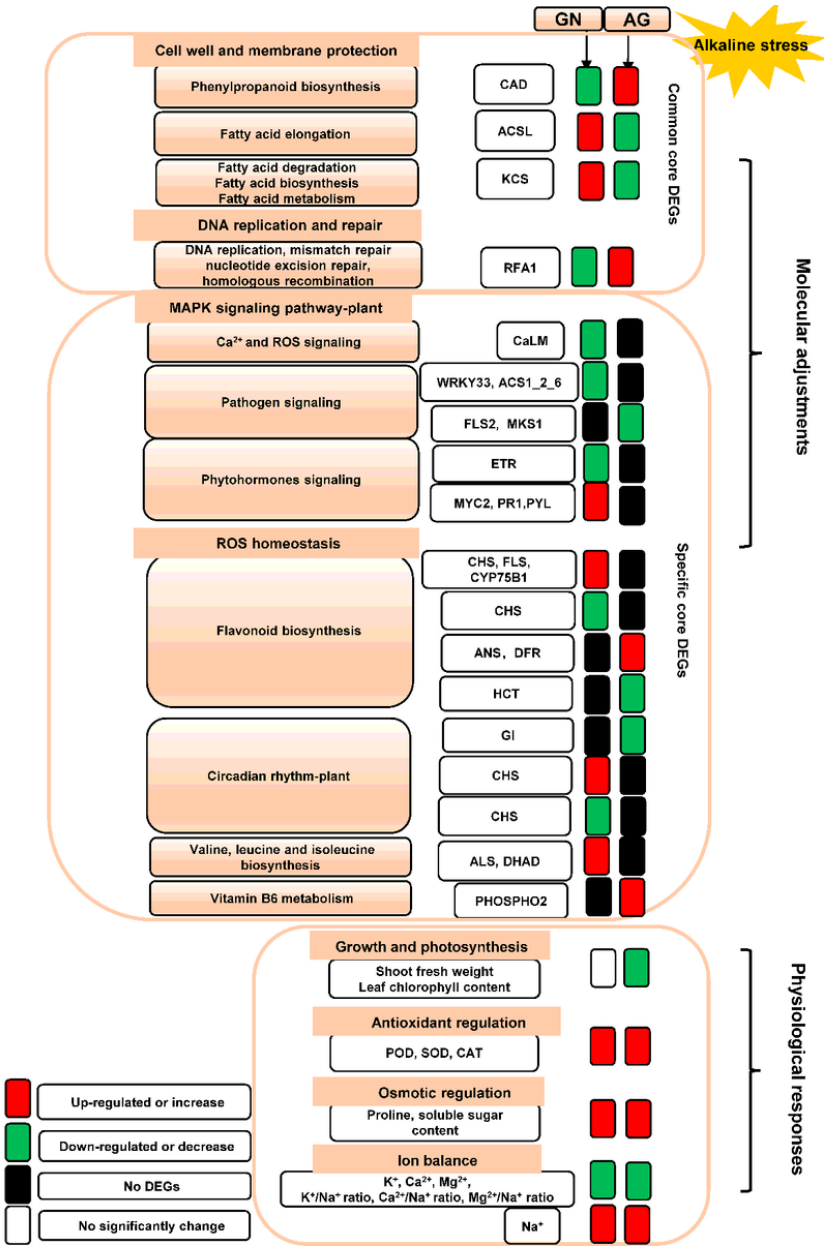



Figure 7

Schematic presentation of the molecular and physiological mechanisms of alfalfa in response to alkaline stress.

## Supplementary Files

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