Comparison of metabolite profiling among model cultivars of wheat, rice and soybean under elevated ozone

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Abstract

As essential source for human consumption, plants of wheat, rice and soybean are highly sensitive to ozone (O3), resulting in significant agricultural losses under O3 pollution. However, little is known about the effects of elevated O3 on their metabolite profiling. In this study, three model cultivars were used for the metabolome analysis under elevated O3 and charcoal filtered air. Our study revealed that wheat and rice differed significantly from soybean in metabolic number and certain pathways. Metabolites response to elevated O3 were less in soybean, whilst those in wheat and rice were considerably larger. Under O3 stress, tricarboxylic acid cycle (TCA) was impaired in three crop plants. Methylerythritol 4-phosphate pathway and glycerol phosphate pathway were altered in wheat and rice with reduced terpene accumulation and high level of phospholipids. However, these pathways were not affected in soybean. Meanwhile, O3 suppressed the generation of flavonoid via benzoic acid pathway in three crop plants. Accordingly, the expressional level of genes coding key enzymes which catalyzed the synthesis or degradation of these metabolites. These findings provide valuable information for understanding of ozone's effects on the metabolite profiling of crop plants, exploring the metabolite differences of three crop plants under elevated O3.

Introduction

Ozone is a double edged sword, protecting life system from the damage of ultraviolet radiation in stratosphere, but generating adverse effects as the concentration rises at the ground level. Among atmospheric pollutants, ground-level ozone (O_3) is considered as one of the most detrimental air pollutants (Fiore *et al* ., 2012; Li & Blande, 2015; Paoletti *et al* ., 2014). Since the 21st century, a strong increase of O_3occurs with an annual average rate of 0.5-2% in the northern mid-latitudes (Stocker, 2014). By 2050, ground-level O_3concentrations may rise 20%-25% globally, with comparable value in India and South Asia by 2020 (Dentener *et al* ., 2005; van Dingenen*et al* ., 2009). Excessive O_3 is not only unhealthy for humans but also detrimental for plants, because elevated O_3 limits growth of plants and affects the yield and quality of crops. It is predicted that the elevated O_3will reduce annual yield for some crops about 26% by 2030 (Avnery*et al* ., 2011). The effect of O_3 on wheat (*Triticum aestivum*) occurs with 6.4-14.9% of yield loss now and this number would rise to 14.8-23.0% by 2020 (Feng *et al* ., 2015). The production of rice (*Oryza sativa*) and soybean (*Glycine max*) are also affected by O_3, with a significant yield decrease of 14% for rice (Ainsworth, 2008) and 28-35% for soybeans in 2020 (Wang & Mauzerall, 2004) compared with that grown in charcoal filtered air. The loss of crop yield results in great economic losses. Annual crop losses, for example, are estimated at about $3-5.5 billion in China and about $2-4 billion in the US due to the damage of O_3, which will increase in the future (van Dingenen *et al* ., 2009).

It is well known that wheat, rice and soybean are the most important crops worldwide. Wheat is depended

on by more than half of the world population (Li *et al* ., 2016; Saitanis *et al* ., 2014; Zhu*et al* ., 2011). Rice is the staple food for the largest number of people on Earth, with total 984 million tons in 2017 (FAO & UNICEF, 2017). Soybean provides vegetable oil for about one third of the world and is also considered as important protein source in Asian countries (Kinney, 1996; Nishinari *et al* ., 2014). However, there is a gap existed between growing demand and crop production. For example, although the increase in yield of wheat by 2% annually until 2020 required for the human beings, high O_3 levels has been accompanied by a loss of production and reduced nutritional value (Biswas *et al* ., 2008; Feng *et al* ., 2008; Singh, Huerta-Espino *et al* ., 2007; Wilkinson *et al* ., 2012). Therefore, understanding the mechanism by which crops respond to increasing O_3 level is pivotal for meeting the increased food demands as the world faces the rapid urbanization, industrialization and climate change.

Previous studies pointed to the negative effects of O_3pollution on crop yield by perturbing the multiple aspects and balance of metabolism in plants (Ainsworth, 2008; Emberson *et al* ., 2009; Li *et al* ., 2018). It is thought that stomata of leaves provide a route for O_3 to enter cells. O_3 causes a range of successive modifications including reduced carbon assimilation and photosynthetic rates, chlorophyll loss, leaf bronzing, development of necrotic spots, senescence and eventually the loss of seed mass and number (Chernikova *et al* ., 2000; Krupa *et al* ., 2001; Baier*et al* ., 2005; Fiscus *et al* ., 2005; Betzelberger *et al* ., 2010). For defense, carbon skeletons diverts to towards distinct pathways contribute to the synthesis of various metabolic compounds, such as flavonoids, phenolic compounds and lignin (Cabané *et al* ., 2004; Kontunen-Soppela *et al* ., 2007; Castagna & Ranieri, 2009; Dizengremel *et al* ., 2012). For instance, accumulation of lignin assists plants to grow erect, facilitates their photosynthesis, and provides protection (Graham *et al* ., 2005).

Previous studies to investigate wheat, rice and soybean in response to O_3 only reveal drastic reductions in the major leaf photosynthetic, thiol-redox state and carbon metabolism proteins and induction of defense/stress-related proteins (Agrawal *et al* ., 2002; Ahsan *et al* ., 2010; Sarkar*et al* ., 2010; Galant *et al* ., 2012). However, comparable assessments of metabolome among the three species have not been examined. O_3 stress affects many pathways, such as mitochondrial respiration, the pentose phosphate pathway, the shikimate and phenylpropanoid pathways, and the anaplerotic metabolic pathway (Dizengremel *et al* ., 2012). Accordingly, the activity of a large number of enzymes in these pathways is also affected by O_3. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisco) activity, for example, showed a significant decrease in the presence of O_3(Sun *et al* ., 2014), while a strong increase in the activity of phosphoenolpyruvate carboxylase (PEPc) also occurs (Landolt *et al* ., 1997; Fontaine *et al* ., 1999; Renaut *et al* ., 2009). This PEPc is thought to participate in anaplerotic CO_2 fixation and could also lead subsequently to different pathways for amino acid synthesis (Melzer & O'Leary, 1987). Although these processes and enzymes in these pathways have been extensively studied, the exact and specific metabolites in these pathways remains to be elucidated in the three species under elevated O_3. The objectives of this study were: 1) to identify components of metabolic composition in wheat, rice and soybean under elevated O_3, 2) to investigate which metabolic pathway was influenced, and their similarities and differences among the three crops.

Material and Methods

Plant material, growth conditions and treatment conditions

Triticum aestivum L. 'Chinese spring', *Oryza sativa* L. 'Japonica', and *Glycine max* L. 'William 82' are model plants of wheat, rice and soybean, for their genomes have been published, respectively (Goff *et al* ., 2002; IWGSC, 2018; Schmutz*et al* ., 2010). The seeds were cultured in the plastic pots of 9 cm in diameter filled with mixture of vermiculite and peat soil. Thirty-day-old seedlings were transferred from culture room to open top chambers (OTCs, height 2.4 m, diameter 2.6 m). The open-top chambers (OTCs) were built and the gas dispensing system was conducted based on the previous method (Uprety, 1998). In this experiment, six chambers were used for two O_3 levels, with three provided with elevated O_3 (EO, 100 ppb) and the other three with charcoal filtered air served as the control (CF, <20 ppb). The injected O_3 was produced by electrical discharge using ambient air by an O_3 generator (CF-KG1, Shanmeishuimei Ltd., Beijing, China). During fumigation period, O_3concentration was monitored above the plant canopy by ozone monitor (Model 106-L, 2B Tech. Inc., USA). According to the record of O_3 monitor, O_3 generator was regulated to maintain

the objective concentration.

Measurements of gas exchange

Gas exchange was determined using flag leaves or the lastest expand leaves treated for 10 days by a gas exchange system (LI 6800). Air flow rate and CO_2 concentration were maintained at 750 μmol s^{-1} and 400 μmol mol^{-1}, respectively. Data including the area-based light-saturated net photosynthetic rate (A sat), stomatal conductance (g s), transpiration rate (E), intercellular CO_2concentration (C i) and ambient CO_2 concentration (C a) were obtained as described previously (Li *et al* ., 2018).

Leaf sampling

After ten days of O_3 fumigation, the most recently expanded leaves on the main stem of the three crops from differential treatments were collected from each chamber per treatment for biochemical measurements. Leaf samples were immediately frozen in liquid nitrogen and then transferred to an ultra-freezer at -80^{o}C until the time of assay.

Determination of activities of antioxidant enzymes and hydrogen peroxide concentration

Leaf samples were homogenized in a pre-chilled mortar and pestle placed on ice with 3 mL potassium phosphate buffer . The homogenate was centrifuged at for 20 min at and the supernatant was used to determine enzymes activities. Superoxide dismutase activity was measured following the method described by Giannopotitis and Ries (Giannopolitis & Ries, 1977). The activity of peroxidase (POD) were analyzed through measuring the oxidation of guaiacol (Rao*et al* ., 1996). Catalase activity was assayed using the method by Aebi (Aebi, 1984). Hydrogen peroxide (H_2O_2) analyses were conducted as described previously (Alexieva *et al* ., 2001).

Metabolite extraction

Leaf samples (0.5 g) were ground in a pre-chilled mortar. The powder was extracted with 50% methanol buffer at room temperature for 10 min followed by storing overnight at -20°C. After centrifugation at 4,000 g for 20 min, the supernatants were transferred into new 96-well plates. The samples were stored at -80°C prior to the liquid chromatograph-mass spectrometer (LC-MS) analysis. In addition, pooled quality control (QC) samples were also prepared by combining 10 μL of each extraction mixture.

Ultra performance liquid chromatography conditions

All samples were analyzed using LC-MS system (ultra performance liquid chromatography (UPLC) system (SCIEX, UK)) following the manufacturer's instructions. The analytical conditions were as follows, UPLC: column, ACQUITY UPLC T3 (100 mm×2.1 mm, 1.8 μm, Waters, UK); solvent system, water (0.1% formic acid) as solvent A, acetonitrile (0.1% formic acid) as solvent B; gradient elution procedures: 0˜0.5 min, 5% solvent B; 0.5˜7 min, 5% to 100% solvent B; 7˜8 min, 100% solvent B; 8˜8.1 min, 100% to 5% solvent B; 8.1˜10 min, 5% solvent B; the injection volume: 4 μL.

Ultra performance liquid chromatography-MS/MS analysis

A high-resolution tandem mass spectrometer TripleTOF5600plus (SCIEX, UK) was used to detect metabolites eluted from the column, operated in both positive and negative ion modes. The parameters were curtain gas 30 pounds per square inch (PSI); Ion source gas1 60 PSI; Ion source gas2 60 PSI; interface heater temperature 650 ; Ion spray voltage 5000 V for positive ion mode, -4,500V for negative ion mode. The mass spectrometry data were acquired in IDA mode. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. The mass accuracy was calibrated every 20 samples during the acquisition with 4 s for dynamic exclusion. A quality control sample (Pool of all samples) was acquired after every 10 samples for evaluating the stability of the LC-MS.

Bioinformatic analysis

The outputs from LC-MS raw data files were converted into mzXML format and then processed using the XCMS, CAMERA and metaX toolbox implemented with the R software. Each ion was identified by combining retention time (RT) and m/z data. A three dimensional matrix containing arbitrarily assigned peak indices (retention time-m/z pairs), sample names (observations) and ion intensity information (variables) was generated and intensities of each peaks were recorded. By matching the exact molecular mass data (m/z) of samples with the online Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB) database, the metabolites were annotated. The molecular formula of metabolites would further be identified and validated by the isotopic distribution measurements, when a mass difference between observed and the database value was less than 10 ppm. Also, an in-house fragment spectrum library of metabolites to validate the metabolite identification was used.

The intensity of peak data was further preprocessed by metaX. Those features detected in less than 50% of QC samples or 80% of biological samples were removed, the remaining peaks with missing values were imputed with the k-nearest neighbor algorithm for improving the data quality. PCA analysis was conducted for outlier detection and batch effects evaluation using the pre-processed dataset. Quality control-based robust LOESS signal correction was fitted to the QC data regarding the order of injection to minimize signal intensity drift over time. The relative standard deviations of the metabolic features were calculated using all QC samples, >30% of which were then removed.

Student t-tests were employed to detect differences in metabolite concentrations between two phenotypes. The P value was adjusted for multiple tests using an FDR -P [?]0.05 (Benjamini–Hochberg). Supervised PLS-DA was conducted through metaX to discriminate the different variables between groups. The VIP value was calculated and a VIP cut-off value of 1.0 was used to select important features.

Quantitative real-time PCR

Total RNA was isolated using RNAiso Plus reagent (TaKaRa) following the manufacturer's instruction. First-strand cDNAs were synthesized from 500-1000 ng of total RNA with PrimeScript RT reagent Kit (TaKaRa) following the manufacturer's instructions. Quantitative RT-PCR was performed using ABI StepOne Plus instrument and SYBR Green Supermix (Bio-Rad) and the primers are listed in Supplemental Table S1. The relative expression levels were calculated against the reference gene by the comparative C_T method (Livak & Schmittgen, 2001).

Results

Gas exchange

We tested the assimilation rate (A sat), stomatal conductance (g s), transpiration rate (E) and the ratio between intercellular carbon dioxide concentration and atmospheric carbon dioxide concentration (C i/C a) of wheat, rice and soybean under charcoal filtered air and elevated [O_3] conditions, respectively. Under O_3 stress condition,A sat, g s and E in wheat were decreased by 32.2%, 19.0%, and 16.2%, while the C i/C a were slightly increased by 3.8%. Similarly, the adverse effect of O_3on A sat, g s, C i/C a and E were suppressed by 49.5%, 65.7%, 7.5% and 60.8% in rice and 19.6%, 52.5%, 9.6% and 47.3% in soybean, respectively (P [?]0.01, Fig. 1).

Effects of O_3 on SOD, POD, CAT and H_2O_2

By comparison, SOD exhibited an increase of activity in wheat, rice and soybean. We only observed significant altered activity of SOD in soybean under O_3 treatment (Fig.2A). Compared with CF treatment, elevated [O_3] inhibited activities of POD and CAT in wheat and soybean, meanwhile, simulated the activity in rice. POD were reduced in wheat and soybean, but increased by two fold in rice (Fig. 2B). There were no significant changes in activities of CAT in rice and soybean, except wheat (Fig. 2C). Moreover, the concentration of H_2O_2 in rice and soybean leaves was strongly increased in response to O_3-exposure (Fig. 2D). However, there was no significant increase in H_2O_2 concentration in wheat.

An overview of the metabolites detected upon O_3treatment

To gain first insights into how O_3 affects the metabolism of crop plants, we performed a comparative metabolic analysis of leaves in wheat, rice and soybean between charcoal filtered air and elevated O_3 treatment. As shown in Table 1, a total of six hundred and forty-four compounds were detected and identified in leaves of the three species. The detected compounds were predominantly grouped into fifty-eight classes, such as, carboxylic acids and derivatives, fatty acyls, prenol lipids, flavonoids, glycerophospholipids, benzene and substituted derivatives.

The score plots based on the model of principal component analysis (PCA) and partial least-squares discriminate analysis (PLS-DA) showed that six repeats in treatment and control group were clustered together and data from two groups could be clearly distinguished in space, respectively (Fig. 3). This suggests smaller system errors during the whole test, data with good repeat ability and differences in metabolites between the two groups for each crop, which enable them for further analysis.

One hundred and ninety-three differential metabolites were found in three species under elevated O_3. Comparative analysis revealed that twenty differential metabolites that overlapped between wheat and rice was higher than the other two comparison sets (four in wheat and soybean, five in rice and soybean). Aspartate was the only one differential metabolite, which was common to the three species and up-accumulated in these species (Fig. 4A). It belongs to the class of carboxylic acids and derivatives, which represent the core metabolome responsive to O_3 stress. The number of soybean-specific differential metabolites was less than that of rice-specific and wheat-specific metabolites.

In wheat, one hundred and four kinds of metabolites were putatively identified, belonging to twenty six kinds of different chemical groups. The relative larger proportions of chemical groups were flavonoids (22.3%), fatty acyls (7.7%), prenol lipids (5.8%), carboxylic acids and derivatives (5.8%), respectively (Fig. 4B). The important and high-fold change metabolites were observed, such as carboxylic acids and derivatives, morphinans, purine nucleotides, glycerolipids and glycerophospholipids (Table S2).

Likewise, eighty-nine metabolites were putatively identified in rice, belonging to twenty kinds of different chemical groups. The relative larger proportions of chemical groups were carboxylic acids and derivatives (30.3%), prenol lipids (10%), flavonoids (8.9%), and fatty acyls (5.6%) (Fig. 4C). Among these metabolites, high fold changes were detected in carboxylic acids and derivatives, glycerophospholipids, quinolines and derivatives (Table S3).

In soybean, twenty-nine differential metabolites were assigned with putative names of identity, belonging to eleven different chemical groups. The relative larger proportions of chemical groups were carboxylic acids and derivatives (37.9%), benzene and substituted derivatives (13.7%), flavonoids (10.3%), prenol lipids (10.3%) and Phenols (6.9%) (Fig. 4D). High fold change metabolites were fatty acyls, phenols, carboxylic acids and derivatives (Table S4).

KEGG enrichment of metabolites

KEGG analysis identified four significant pathways related with amino acids (alanine, cyano amino acid, phenylalanine, alanine aspartate and glutamate) in wheat. Similar with wheat, metabolisms associated with amino acids (Alanine, phenylalanine, cysteine, methionine and pyrimidine) and nucleotides (purine and pyrimidine) were significantly enriched in rice. These results suggest that O_3 affects the synthesis of certain amino acids in wheat and rice. In soybean, in addition to amino acid metabolism, the biosynthesis of alkaloids and terpenoids were also altered by O_3 (Fig. 5).

Analysis of metabolite pathway in leaf exposed to O_3

The activity of cytosolic pyruvate kinase (PK), NAD-malic enzyme and fumarase in mitochondria were induced after exposure to ozone (Dizengremel *et al* ., 1994; Gerant*et al* ., 1996; Gaucher *et al* ., 2006; Dizengremel *et al* ., 2009). The changed metabolites in tricarboxylic acid (TCA) cycle under O_3 treatment were isocitrate with increased 2.7 fold change in rice (Fig. 7, Table S3), 2-oxoglutarate with a reduction of aproximately 50% and citric of 50% in soybean (Fig. 8, Table S4). Additionally, O_3 treatment leads to the high level of aspartate-derived asparagine or aspartate in wheat, rice and soybean. Subsequently, the level

of arginine in soybean and agmatine in wheat are elevated in ornithine cycle. These results suggest that O_3 affects TCA cycle in three crop plants and ornithine cycle in wheat andsoybean .

In mevalonate pathway, phosphoenolpyruvate is used for the synthesis of isoprenoids that play an antioxidant role and prevent photosynthetic electron transport damage (Loreto & Velikova, 2001; Magel *et al* ., 2006; Vickers *et al* ., 2009). The decreased level of sterols in wheat but the increased concentration in rice were observed under O_3 stress (Fig. 6 and 7). Moreover, chloric O_3 leads to the declined concentration of fatty acids, monoteroenes, sesquitepenes, saponins, diterpenes, terpene and polyterpenes in both wheat and rice. However, all these metabolites were not changed in soybean in response to O_3 (Fig. 8).

Futhermore, in the glycerol phosphate pathway and pentose phosphate pathway, O_3 motivated the synthesis of purines in the three species, and phospholipids only in wheat and rice. On the contrary, histidine was up-regulated in soybean, but not changed in wheat and rice.The shikimate pathway is also affected by O_3 stress. In wheat, N-acetyltryptophan was highly accumulated with 10 fold, while levels of indole alkaloids, methyl salicylate, cinnamic acid, lignin, phenylpropanoids, flavonoids, and hydroxybenzoic acid were reduced in various extent (Fig. 6, Table S2). These metabolites are known to be involved in thickening of cell walls and also act as antimicrobial compounds (Yogendra *et al* ., 2015). In rice, metabolites of cinnamic acid, coumaric acid, flavonoids, salicylate, and indole alkaloids showed reduced abundance, while phenylalanine and kynurenic acid were increased (Fig. 7). In soybean, several products of 3-(4-hydroxyphenyl)pyruvate, 4-coumarate and flavonoids were reduced but methylsalicylate, lignin, phenylpropanoids and 4-hydroxybenzoic acid were elevated (Fig. 8).

Quantitative real-time PCR (qRT–PCR) analysis

Citrate synthase (CS) is generally considered as the rate-limiting enzyme of tricarboxylic acid cycle, whose reduction may lead to an decreased availability of citrate but increased availability of oxaloacetate (van Ooyen *et al* ., 2012). The presence of transcript levels by qRT-PCR revealed a 3-fold increase in wheat and a 2-fold in rice for citrate (Si)-synthase by O_3 (Fig. 9A). However, the transcript levels of citrate (Si)-synthase and ATP citrate synthase were drastically declined by approximately 64% and 90% in soybean under elevated O_3 compared with control, respectively (Fig. 9A, B). The oxidative decarboxylation of isocitrate to α-ketoglutarate was catalyzed by mitochondrial NAD^+-specific isocitrate dehydrogenase (NAD^+) (Anderson *et al* ., 2000), which was strongly enhanced by O_3 in wheat (3.2 fold), rice (1.8 fold) and soybean (6.8 fold) (Fig. 9C). Oxoglutarate dehydrogenase (succinyl-transferring) is responsible for the conversion of 2-oxoglutarate to succinyl-coenzyme A (Araújo *et al* ., 2008), whose expression is highly increased in three crop plants with the expression being highest (3.1-fold change) in soybean (Fig. 9D). It has been reported that L-glutamate and oxaloacetate are generated by the enzyme of aspartate transaminase in the presence of α-ketoglutarate and L-aspartate (Brauc *et al* ., 2011; Han *et al* ., 2011). It was exhibited that O_3 led to a significantly accelerated induction of aspartate transaminase in three crop plants (Fig. 9E).

Discussion

The major aim of the present study was to assess the effect of elevated [O_3] on the metabolites in different crop species which is little known so far. Wheat, rice and soybean feed more than half of the world population, yet they are the most sensitive crops to O_3 pollution. Previous model studies predicted their huge yield loss under elevated [O_3] in the past 20 years (Sitch *et al* ., 2007; Avnery *et al* ., 2011b). Although many papers demonstrated the damage of O_3 to crops, few researches were performed to compare the adverse effecs of O_3 on different species, especially between monocots and dicots. Therefore, understanding how elevated [O_3] affects crops via metabolic pathway is crucial for efforts to imporve their performance. Here, we found that some metabolic changes were common to the three species, whereas others were specific in monocots (wheat and rice) or dicots (soybean).

Photosynthesis is the primary source of chemical energy and biomass accumulation, it is therefore essential for plant growth and development (Kruse *et al* ., 2005; Melis, 2013). Previous findings reported that chronic exposure to O_3impaired stomata conductance and photosynthesis in plant leaves through change of calcium influxes and increased photorespiration (Ainsworth, 2008; Avnery*et al* ., 2011a). Consistent with this,

CO_2assimilation rates (A_{sat}), stomatal conductivity (g s) and transpiration rate (E) were reduced in wheat, rice and soybean under O_3 stress in our study. It has been observed that the reduction of photosynthesis coupled to alteration of cellular metabolism (Dizengremel, 2001; Heath, 2008; Reich, 1983). We found the pentose phosphate pathway was disturbed by O_3 in three crop plants with highly increased concentration of the purines, which are converted from Ribulose-1,5-bisphosphate carboxylase/oxygenase (Ribulose D-Ribulose-5-P). The glycerol phosphate pathway was impaired only in wheat and rice by O_3, leading to the increased concentration of phospholipids. This metabolic trait seems to be associated with different species in response to O_3. Subsequently, the shikimate-phenylpropanoid pathway starting in chloroplasts was affected in three crop plants by O_3.In this pathway salicylate-derived methyl salicylate (SA) was generously generated in soybean, while the concentration of salicylate was reduced in rice and wheat. SA and methylated salicylic acid (Me-SA) have been reported to be required for adaptive responses to certain biotic and abiotic stresses (Rekhter *et al* ., 2019). Furthermore, low concentrations of SA generally enhance the antioxidant capacity in plants, but high concentrations of SA may cause cell death or susceptibility to abiotic stresses (Vlot *et al* ., 2009). These results implied that SA or Me-SA is involved in the regulation of O_3 response but it functions in different manner in monocots as wheat and rice, and dicots as soybean. These two types of plants may evolved different strategies to minimize the adverse effects of O_3 stress. Similarly, the synthesis of flavonoids, lignin and phenylpropanoids also altered in this pathway. These metabolites are induced by various adverse environmental stresses (Tzin & Galili, 2010). The study presented here pointed to increased concentration of the lignin and phenylpropanoids induced by O_3 in soybean, which was consistent with that in poplar (Cabané *et al* ., 2004). On the contrary, the level of lignin and phenylpropanoids in wheat was decreased. It suggests that stimulation of enzyme activities by O_3 involved in the more specific lignin pathway is different according to the different species. Previous studies showed that the change of lignin biosynthesis could alter the synthesis of other secondary metabolites (Baxter & Stewart, 2013). Comparable reduction in flavonoids occured across wheat, rice and soybean, suggesting that the three species share same metabolic pathway leading to the reduced synthesis of flavonoid in response to O_3. This was strongly associated with the decrease of cinnamic acid. Flavonoids, as an effective abiotic elicitor, not only showed significant positive correlations with seed yield but also act as photoprotective compounds and antioxidants (Mao *et al* ., 2017; Middleton & Teramura, 1993). To this end, flavonoids may play an important regulatory role in the seed yield rather than protecting plants against elevated [O_3].

Previous studies reported that O_3 entered leaves of plants primarily via stomata, and then generated reactive oxygen species (ROS), such as superoxide $O_2^{\cdot-}$, single oxygen, hydroxyl radicals and hydrogen peroxide in plant cells, which can destroy the structure of DNA, proteins, lipids and carbohydrates due to oxidation capacity (Vaultier & Jolivet, 2015). In the study, O_3 induced significant increase of H_2O_2 in rice and soybean. In order to resist O_3-induced oxidative stress, plants have elvolved various mechanism to avoid detrimental reactions, such as motivate activities of antioxidant enzymes (Vendruscolo *et al* ., 2007). The activities of SOD and POD have been considered as indicators for eliminating oxidative stress and scavenging ROS (Vendruscolo *et al* ., 2007). Our data demonstrated O_3 up-regulated activities of SOD, POD and CAT in rice, suggesting rice utilized high activities of these antioxidant enzymes as a strategy to detoxify O_3-induced stress. However, activities of POD and CAT reduced in soybean but H_2O_2 was increased with great extant after O_3 exposure. This suggests that the O_3-induced oxidative stress is beyond the ability of this antioxidant enzyme to detoxify ROS (Biswas *et al* ., 2008). The elevated SOD activity was accompanied with decreased activities of POD and CAT in wheat, indicating these antioxidants can compensate for each other in removing ROS toxicity.

Damage of mitochondrial respiration generated ROS when plants were fumigated by O_3 (Dizengremel *et al* ., 2012). NADH, which is produced by the photorespiration-associated glycine decarboxylase (GDC) in the mitochondrion, induces retroinhibition on the decarboxylating enzymes of the TCA cycle (Igamberdiev & Gardeström, 2003). The TCA-cycle is one of the important metabolic pathways (Dizengremel *et al* ., 2012), which aims to produce energy to sustain plant growth. In this study, TCA cycle was impaired in both rice and soybean under O_3 stress. However, the intermediate product changes were not consistent in rice (isocitrate) and soybean (citrate and 2-oxoglutarate). Moreover, the expression of genes coding citrate-synthase and

isocitrate dehydrogenase was down-regulated in soybean treated with elevated O_3, providing a reasonable explanation for the reduced accumulation of citrate and 2-oxoglutarate. Meanwhile, the expression of genes coding citrate (Si)-synthase in rice was significantly enhanced by O_3. This increased citrate may be largely converted to isocitrate in rice. The change in TCA-cycle intermediates in the two species may serve as an energy conservation fashion to cope with O_3 stress. Moreover, it also indicates that the soybean as dicot and rice as monocots may evolved different strategies to minimize the adverse effects of O_3 stress. Subsequently, these changes lead to the high level of aspartate-derived asparagine or aspartate in wheat, rice and soybean. However, the elevated expression of genes coding aspartate transaminase might create a negative feedback loop in this metabolic pathway. Previous studies proved that asparate is the central regulator between carbon and nitrogen metabolism (Less & Galili, 2008). Carbon and nitrogen are the two most essential elements for plant growth and development, especially for crop productivity and quality. Therefore, coordinted carbon and nitrogen metabolism regulates plant development and metabolic in responding to varied environmental conditions (Kang & Turano, 2003). In this study, the significant O_3-induced accumulation of aspartate implies that maintaining cellular carbon/nitrogen balance was an important metabolic mechanism and these amino acids are important for the adaptation of the plants to O_3 conditions. Aspartate-semialdehyde dehydrogenase is a control point in isoleucine, methionine, lysine, and threonine synthesis (Schroeder *et al* ., 2010). In the present study, the production of methionine in rice was significantly enhanced. Pyrimidines converted by glutamine were elevated in rice and wheat rather than in soybean, which coincied with the SOD, POD and CAT facilitating the regeneration of redox ascorbate and glutathione metabolites (Foryer & Noctor, 2000). Arginine acts as a storage form of organic nitrogen due to its highest nitrogen/carbon ratio. It has been reported that accumulation of arginine attenuated oxidative stress in higher plants under salinity stress (Qados, 2010; Winter *et al* ., 2015). In this study, arginine in soybean and derived-arginine agmatine in wheat were strongly enhanced under elevated [O_3]. These findings indicate a high demand for nitrogen release during O_3 stress.

Collectively, the metabolic response of plants to O_3stress is currently receiving more attention. This is driven by global crop supply facing challenge from continuous growth of population, agricultural land lost and climate change, especially O_3pollution (Stocker *et al* ., 2013). Our current data suggests that elevated [O_3] induces a wide range of changed metabolites in three species which contribute to avoiding O_3 stress. It is also found that oxidative damage induced by O_3 leads to the change of activity in key metabolic enzymes of different species. Together, these results have direct implications for different crop improvement strategies. Ultimately, understanding how environmental O_3 affects metablolic pathways in different plants provides theoretical foundation for improving agronomic traits of crop plants under the environment of global climate change, specially O_3 pollution.

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Author contribution

CL and TW planned and designed the research. XZ, YZ, DZ, XG, YZ, and LZ performed the experiment, conducted fieldwork, and analysed data. XZ, CL, and TW wrote the manuscript. CL and TW contributed equally.

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Table

Table 1 Classification of the 644 detected metabolites in wheat, rice and soybean accessions into major classes

Groups	**Number of Compounds detected**	**Groups**	**Nu**
Carboxylic acids and derivatives	135	Alcohols and polyols	2
Fatty acyls	77	Benzopyrans	2
Prenol lipids	57	Indanes	2
Flavonoids	54	Naphthofurans	2
Glycerophospholipids	31	Tetrapyrroles and derivatives	2
Benzene and substituted derivatives	23	Unsaturated hydrocarbons	2
Organooxygen compounds	19	Coumarins and derivatives	2
Glycerolipids	18	Pyrenes	2

Groups	Number of Compounds detected	Groups	Nu
Purine nucleosides	14	Oxanes	1
Cinnamic acids and derivatives	11	Oxepanes	1
Pyrimidine nucleosides	8	Isobenzofurans	1
Sterol lipids	8	Macrolides and analogues	1
Indoles and derivatives	7	Morphinans	1
Hydroxy acids and derivatives	6	Nucleosides, nucleotides, and analogues	1
Phenols	6	Phenylpropanoic acids	1
Organonitrogen compounds	5	Saccharolipids	1
Isoflavonoids	5	Tannins	1
Naphthalenes	4	2-arylbenzofuran flavonoids	1
Diazines	4	5'-deoxyribonucleosides	1
Quinolines and derivatives	4	Amines	1
Sphingolipids	4	Azoles	1
Anthracenes	3	Benzodioxoles	1
Keto acids and derivatives	3	Benzothiazoles	1
Lactones	3	Cinnamaldehydes	1
Furans	3	Dioxepanes	1
Piperidines	3	Ethers	1
Imidazopyrimidines	3	Peptidomimetics	1
Pyridines and derivatives	3	Tetrahydroisoquinolines	1
Pteridines and derivatives	3	Null/unknown	84

Figure legends

Fig.1 Effects of elevated [O_3] on CO_2 assimilation rate (A_{sat}) (A), stomatal conductance (g_s) (B), ratio of CO_2 concentration in intercellular and ambient (C_i/C_a) (C) and transpiration rate (E) (D) of wheat, rice and soybean. Columns in the figure represent means ± SE (n =6) and two asterisks (**) on bars indicate significant differences between charcoal filtered air (CF) and elevated [O_3] (EO) treatment for each crop at the 0.01 level.

Fig. 2 Effects of elevated [O_3] on activities of superoxide dismutase (SOD) (A), peroxidase (POD) (B), and catalase (CAT) (C) and hydrogen peroxide (H_2O_2) (D) in wheat, rice and soybean leaves. Columns in the figure represent means ± SE (n =3). One asterisk (*) and two asterisks (**) on bars indicate significant differences at the 0.05 and 0.01 level between charcoal filtered air (CF) and elevated [O_3] (EO) treatment for each crop, respectively.

Fig. 3 Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) for the three crops. A: PCA analysis between sample groups. B-D: PLS-DA score plots of the metabolite profiles of different crops in treatment and control groups. B: Tae, wheat; C: Osa, rice; D: Gma, soybean.

Fig. 4 Differential metabolites. (A) The common or specific different metabolites in three species under elevated O_3. (B-D) Classification of differential metabolites under O_3 stress according to chemical groups identified in three species wheat (B), rice (C) and soybean (D).

Fig.5 KEGG enrichment analysis of differential metabolites in wheat, rice, and soybean (P <0.05). Tae, wheat; Osa, rice; Gma, soybean.

Fig. 6 Overview of metabolite changes in wheat exposed to O_3. A schematic view of different metabolic pathways in which metabolites were affected by O_3 is shown. The increased metabolites are marked in red and the decreased onesare marked in blue . Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 3PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; HMP, hexose monophosphate pathway; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid; TCA, tricarboxylic acid cycle; IPP, isopentene

pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranylpyrophosphate; FPP, farnese pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

Fig. 7 Overview of metabolite changes in rice exposed to O_3. A schematic view of different metabolic pathways in which metabolites were affected by O_3 is shown. The increased metabolites are marked in red and the decreased ones are marked in blue. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 3PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; HMP, hexose monophosphate pathway; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid; TCA, tricarboxylic acid cycle; IPP, isopentene pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranylpyrophosphate; FPP, farnese pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

Fig. 8 Overview of metabolite changes in soybean exposed to O_3. A schematic view of different metabolic pathways in which metabolites were affected by O_3 is shown. The increased metabolites are marked in red and the decreased ones are marked in blue. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 3PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; HMP, hexose monophosphate pathway; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid; TCA, tricarboxylic acid cycle; IPP, isopentene pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranylpyrophosphate; FPP, farnese pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

Fig. 9 The expression level of genes coding key enzymes. Columns in the figure represent relative expression ± SE of three biological samples. A: Rhe gene coding citrate (Si)-synthase (EC2.3.3.1); B: The gene coding ATP citrate synthase (EC2.3.3.8); C: The gene coding isocitrate dehydrogenase (EC1.1.1.41); D: The gene coding oxoglutarate dehydrogenase (EC1.2.4.2); E: The gene coding aspartate transaminase (EC2.6.1.1).

Supporting Information

Table S1 Primers used in this study.

Table S2 Identified metabolites whose concentration showed strongly significant differences between elevated O_3 and control treatment in wheat (fold change[?]2 and [?] 0.5, P <0.05).

Table S3 Identified metabolites whose concentration showed strongly significant differences between O_3 stresses and control in rice (fold change[?]2 and [?] 0.5,P <0.05).

Table S4 Identified metabolites whose concentration showed strongly significant differences between O_3 stresses and control in soybean (fold change[?]2 and [?] 0.5,P <0.05).

Fig. S1 Hierarchical clustering analysis (HCA) of differentially accumulated metabolites in wheat, rice, and soybean under elevated O_3. Hierarchical trees drawn were based on detected metabolites in leaves of wheat, rice and soybean in charcoal filtered air (CF) condition and elevated [O_3] (EO) treatment. Columns represent different crops at different conditions and rows indicate different metabolites.