Fertilization of Grapevine Based on Gene Expression

Cheng Zhang, Haifeng Jia, Jingjue Zeng, Tariq Perraiz, Zhenqiang Xie, Xudong Zhu, and Chen Wang*

Abstract
The application of genetic information in agricultural production is an important issue, which is highly worthy of attention. Gene expression data can accurately reflect the growth and metabolic status of plants, with which we can predict and monitor the nutritional requirements of plants and then derive accurate fertilization strategies. In this study, to verify the feasibility and workability of gene information-based fertilization strategies and to figure out the specific nutritional requirements of grapevine (Vitis vinifera L.) at various developmental stages, the expression levels of 13 N–P–K uptake and metabolism genes and their responses to fertilization during the flowering and berry development stages were validated by using quantitative polymerase chain reaction (PCR). The results showed that in the particular stages where N–P–K uptake and metabolism genes were highly expressed, these genes also showed more positive responses to fertilization and the grape quality was more dramatically improved. This proved the feasibility and workability of this novel fertilization strategy. The nutritional requirements of grapevine during the flowering and berry developmental phases were summarized in terms of gene expression levels, in which grapevine needs more P at the flowering stage, more N at the first berry expansion stage, less nutrient at the seed stone hardening stage, and more P and K at the second berry expansion stage and the veraison stage. The present study is one of the novel and initial findings regarding the application of fertilizers in vineyards for better cultivation of grapevine during common cultural practices.

Core Ideas
- This approach is more accurate and reliable than traditional fertilization strategies.
- This study verified the feasibility and workability of gene expression-based fertilization strategies.
- The specific nutritional requirements of grapevine at different developmental stages were depicted in the level of gene expression.

GRAPEVINE is one of the world’s most economically important fruit crops (He, 1998; Kong, 2004). It is highly responsive to the application of fertilizers and has been reported as a heavy feeder of N, P, and K. Fertilization of the soil is one of the most important viticulture techniques, with a great effect on the vineyard yield and the grape quality. Fertilizer application usually results in increased yield; however, excessive or unbalanced applications can have negative effects on the yield and fruit quality. Traditional fertilization strategies are dominantly based on phenology and personal

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Abbreviations: FE, fertilization; SC, sample collecting; VvAS, V. vinifera asparagine synthetase; VvGDH, V. vinifera glutamate dehydrogenase; VvGS, V. vinifera glutamine synthetase; VvNiR, V. vinifera nitrite reductase; VvNR, V. vinifera nitrate reductase; VvKUP1, V. vinifera K⁺ uptake and metabolism permeases 1; VvKUP2, V. vinifera K⁺ uptake and metabolism permeases 2; VvSIRK, V. vinifera inward rectifying shaker-like K⁺ channel; VvSORK, V. vinifera outward rectifying shaker-like K⁺ channel; VvPAP, V. vinifera purple acid phosphatase; VvPHO1, V. vinifera phosphate (P) transporter 1; VvPHT1–4, V. vinifera phosphate transporter 1–4; VvPHT2–1, V. vinifera phosphate transporter 2–1

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experiences, which are regional and might be influenced by various environmental factors such as temperature and precipitation (Moll et al., 1982; Ehlenk & Kirchner, 2002). In general, the inaccuracy and lack of universality of traditional fertilization strategies are not meeting the rapid development of precise agriculture, which calls for a more accurate and reliable fertilization guidance. This approach could help to meet the requirements of precise agriculture, which is now needed to cope with the rapid growth of the world population.

Gene expression results exclusively from the interaction of factors within plants and environmental conditions and reflects the real growth and metabolic states of the crops. Moreover, gene expression occurs earlier than the specific morphological changes of crops, which can further predict or prediagnose the nutritional requirements, diseases, and damage caused by adverse environmental conditions such as drought, waterlogging, and hypersalinity. Following this principle, Wang et al. (2014a) depicted grapevine phenology by analyzing the expression profiles of nine grapevine flower and berry development genes and verified the workability by fertilization trials. The same principle has also been widely applied in the field of medicine to prediagnose some diseases in humans (Khan et al., 2001; Tibshirani et al., 2002; Wright et al., 2003).

With the full genome of grapevine being sequenced and released publicly in 2007 (Jaillon et al., 2007; Velasco et al., 2007), grapevine has evolved as a model plant for molecular biology and genomic studies among fruit crops. This provides novel insights for solving problems encountered in grapevine production and for promoting the development of a precise grapevine industry. Applying fertilizers during flowering and berry developmental stages is a direct and efficient way to improve the yield and quality of grapes. However, grapevine at different developmental stages has different nutritional requirements; application of fertilizers at inappropriate stages may be inefficient, cause wastage, and even negatively affect plant growth and grape production.

The grapevine N metabolism genes VvNR (V. vinifera nitrate reductase), VvNIR (V. vinifera nitrite reductase), VvGS (V. vinifera glutamine synthetase), VvGDH (V. vinifera glutamate dehydrogenase), and VvAS (V. vinifera asparagine synthetase) encode the key enzymes in the process of N assimilation (Hirel et al., 2001; Lam et al., 1996; Wickert et al., 2007). Grapevine K uptake genes VvKUP1 (Vitis vinifera K+ uptake and metabolism permeases 1), VvKUP2 (V. vinifera K+ uptake and metabolism permeases 2), VvSIRK (V. vinifera inward rectifying shaker-like K+ channel), and VvSORK (V. vinifera outward rectifying shaker-like K+ channel) and P uptake genes VvPHT1–4 (V. vinifera phosphate transporter 1–4), VvPHT2–1 (V. vinifera phosphate transporter 2–1), VvPHO1 (V. vinifera phosphate (P) transporter 1), and VvPAP (V. vinifera purple acid phosphatase) all play important roles in the absorption and transportation of K and P in grapevine (Davies et al., 2006; Hamburger et al., 2002; Johansson et al., 2006; Pratelli et al., 2002; Shin et al., 2004; Tong et al., 2005; Wang et al., 2014b). Their transcriptional activities can reflect the basic acquisition and metabolic status of N–P–K nutrients and thus their expression information can be used to depict the nutritional requirements of grapevine and provide guidelines for grapevine fertilization. During the growth and development period of grapevine, the higher the N–P–K uptake and metabolism genes are expressed, the more actively the N–P–K elements are absorbed, transported, and metabolized, indicating that more N–P–K nutrients are needed by grapevine. Thus applying fertilizers at the stages where the N–P–K uptake and metabolism genes are highly expressed will achieve better results. To verify this hypothesis, fertilization experiments were performed and the responses of these N–P–K uptake and metabolism genes to fertilization and changes in the grape quality parameters were measured. This study aims to elucidate the feasibility and workability of gene expression based-fertilization strategies, which might be helpful in developing effective, low-cost precision agronomy and reveal a new opportunity for the application of gene information in grapevine production.

**Materials and Methods**

**Plant Materials**

The experimental samples of flowers and berries at different developmental stages were collected from rooted 5-yr old ‘Summer Black’ grapevines (a V. vinifera × Vitis labrusca L. hybrid) that were grown under standard cultivation conditions under a rain shelter at Fujiabian Agriculture Technological Sightseeing Garden, Nanjing, Jiangsu Province, China. The soil type is a sandy soil with a pH value of 6.7, 96 mg kg⁻¹ available N, 35 mg kg⁻¹ available P, 206 mg kg⁻¹ available K. Samples were collected every 6 d from 4 May 2014 (early flowering stage) to 30 June 2014 (late veraison stage), at 9:00 to 9:30 AM. At the first and second and third to tenth stages, flowers and berries were sampled, respectively. After collection, all samples were frozen immediately in liquid N and stored at −80°C until used for further investigation.

**Design of Fertilization Assay**

Fertilizers application was performed at 6-d intervals from 4 May (early flowering stage) to 30 June (late veraison stage). Urea (50 g per plant) and KH₂PO₄ (100 g per plant) were applied to grapevine plants. For each treatment, three replicates (each replicate was one grapevine plant) were used. Another three grapevines under the same cultivation conditions without treatment were kept as controls. After application, the treated regions were covered in plastic mulch to avoid loss of fertilizer by dissipation, runoff, etc. Samples were collected at three continuous stages (3, 6, and 9 d after every fertilization treatment; Table 1). The first and second treatments (FE-1 and FE-2) had flowers and the third to tenth (FE-3 to FE-10) treatments had berries sampled. After collection,
all samples were frozen immediately in liquid N and stored at −80°C until use.

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted following the sodium dodecyl sulfate–phenol method modified by Zhang et al. (2010a). RNA purity and integrity were assessed by the A260/A280 absorbance ratio and 1.0% agarose gel. The concentration of total RNA was measured according to the A260 absorbance after genomic DNA had been digested by deoxyribonuclease. First-strand cDNA was synthesized from 4 μg of DNA-free RNA using a RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD). The cDNA was diluted 10 times.

**Real-Time PCR and Data Analysis**

The real-time quantitative PCR reaction solution was comprised of 10.0 μL of SYBR Premix Ex Taq (Takara, Dalian, Japan), 0.4 μL of each primer (10 μM), 2 μL of cDNA, and 7.2 μL of RNase-free water in a total volume of 20 μL. The reaction was performed in a Light Cycler 1.5 instrument (Roche, Basel, Germany), started with a preliminary step of 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 61°C for 20 s. A template-free control for each primer pair was set for each cycle. The details of primers are shown in Table 2. All PCR reactions were normalized using the cycle threshold value corresponding to the grapevine actin gene. Three biological replications were used and three measurements were performed on each replicate. For the fertilizer-treated samples, expression levels produced by the quantitative PCR were expressed as a ratio relative to their corresponding controls, which were set to 1. The significant differences of the results were analyzed statistically using SPSS version 15.0 (IBM SPSS, Chicago, IL) at 0.05, 0.01, and 0.001 (P-value) levels.

**Measurement of Fruit Quality Traits**

Ripe grape berries were collected from treated and the corresponding control plants at 9:00 AM on 20 July. Fruit quality indices including single grain weight, cluster weight, total soluble solids, and total sugar content were measured. For each treatment, at least 10 clusters of grape samples were weighed to calculate the average cluster weight and 20 berries per cluster were used to calculate the average single grain weight and then used to determine the soluble solids concentration with portable a total soluble solid concentration detector (PN007529, Yacite, Beijing, China) according to the operating instructions. Total sugar content was performed by high performance liquid chromatography (Merck Hitachi, Darmstadt, Hesse, Germany). Quantification of samples was accomplished by comparison to authentic standards (Sigma-Aldrich Chemie, Steinheim, Germany). Three samples from 20 berries were randomly collected from each treatment and then juice was extracted. Three replicates per treatment were centrifuged at 20,000g for 13 min at 4°C. The supernatant was filtered through Sep-Pack Cartridges (Waters, Taunton, MA) and then again through a 0.45-μm membrane filter (Milllex HV13,
Table 2. Primers used for quantitative real-time polymerase chain reaction (RT-PCR).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO1</td>
<td>GCAAGGCATGCAATGCTAATTGACTAATC30VN†</td>
<td>cDNA synthesis</td>
</tr>
<tr>
<td>PO2</td>
<td>GACAGTGTTGGATACGCCAGATGACGGG</td>
<td></td>
</tr>
<tr>
<td>Actin For‡</td>
<td>TCACCACACTGCTGTAACG</td>
<td>Internal control</td>
</tr>
<tr>
<td>Actin Rev</td>
<td>CTTTGCAGGACAGAATTG</td>
<td></td>
</tr>
<tr>
<td>GDH For</td>
<td>TGAATGCTGTCGAAAGCACCAC</td>
<td>VvGDH quantitative RT-PCR</td>
</tr>
<tr>
<td>GDH Rev</td>
<td>TAGGGTCCTCCTCAACCTAAAAGG</td>
<td></td>
</tr>
<tr>
<td>NR For</td>
<td>ATGGGCTCTCCTCCCTGTC</td>
<td>VvNR quantitative RT-PCR</td>
</tr>
<tr>
<td>NR Rev</td>
<td>TCAGTCCTGCTCCTCTTCCCT</td>
<td></td>
</tr>
<tr>
<td>GS For</td>
<td>ATGACACACTAAGGAGGGT</td>
<td>VvGS quantitative RT-PCR</td>
</tr>
<tr>
<td>GS Rev</td>
<td>TCAAATCTGAGGAAGCC</td>
<td></td>
</tr>
<tr>
<td>AS For</td>
<td>ATGTCGGGAACATCTGACTGCTGGA</td>
<td>VvAS quantitative RT-PCR</td>
</tr>
<tr>
<td>AS Rev</td>
<td>GGATTGGATACCCCTGACATGG</td>
<td></td>
</tr>
<tr>
<td>PHT1–4 For</td>
<td>GTACTCTGCGCGAGCATCTC</td>
<td>VvPHT1–4 quantitative RT-PCR</td>
</tr>
<tr>
<td>PHT1–4 Rev</td>
<td>GCCGAGAATTGGAAAGGGAG</td>
<td></td>
</tr>
<tr>
<td>PHT2–1 For</td>
<td>CAGGCGCTTAACTCCTGAGAG</td>
<td>VvPHT2–1 quantitative RT-PCR</td>
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<tr>
<td>PHT2–1 Rev</td>
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</tr>
<tr>
<td>PHO1 For</td>
<td>ACCCAAAGGTGACCCATT</td>
<td>VvPHO1 quantitative RT-PCR</td>
</tr>
<tr>
<td>PHO2 For</td>
<td>GACAGACATGATTTGACGGAT</td>
<td></td>
</tr>
<tr>
<td>PAP For</td>
<td>ATCCGATATATTGCTCTCCGG</td>
<td>VvPAP quantitative RT-PCR</td>
</tr>
<tr>
<td>PAP Rev</td>
<td>TCCGATTTCTCCGAGCTTAC</td>
<td></td>
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<tr>
<td>SIRK For</td>
<td>GCTTCTAGCTCCTGTGTC</td>
<td>VvSIRK quantitative RT-PCR</td>
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<tr>
<td>SIRK Rev</td>
<td>CACCAACTAGGTTGCTT</td>
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<tr>
<td>SORK For</td>
<td>AGAGGACGACAGGAGAAGAGAA</td>
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<td>SORK Rev</td>
<td>CACAGAGTCATCATGTTCAA</td>
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<tr>
<td>KUPI For</td>
<td>TGAGCTTGTGCAACATGGAAAGACT</td>
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<td>KUP2 For</td>
<td>ATGGCTCTCCTGCCCATCTCACA</td>
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</tr>
<tr>
<td>KUP2 Rev</td>
<td>GCTGTCATGTGATTTGATGTCG</td>
<td></td>
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</table>

† T30VN: 30 repetitive T; ‡ V indicates A, G, C, or T.

Milipore Corp, Boston, MA). The high performance liquid chromatography for sugar analysis was equipped with a pump (Model L-7100, Merck Hitachi), fitted with an amino column (250 × 4, NH2; 5 μm particle size, Merck, Darmstadt, Germany). Aliquots of 20 μL were applied via an auto sampler (Mod LaChrom 7200, Merck Hitachi). The mobile phase used was acetonitrile/water (85:15, v/v) at a flow rate of 1.5 mL min−1 and analyzed using refractive index detection (Mod LaChrom 7490, Merck Hitachi). All analyses were performed in three replicates for each sample. The results were analyzed statistically using SPSS version 15.0 and the value of p < 0.05 was considered statistically significant.

Results

Expression Analysis of N–P–K Uptake and Metabolism Genes during the Flowering and Grape Berry Developmental Periods

Five N assimilation genes, four P uptake genes, and four K uptake genes of grapevine were selected to evaluate gene expression profiles during the flowering and berry developmental periods. Grapevine samples (flowers and berries) collected at 10 growth stages (every 6 d from 4 May to 30 June) were subjected to quantitative real-time PCR analysis. Data analysis depicted that the expression of these 13 N–P–K uptake and metabolism genes showed different expression peaks during flowering and berry development. The five N assimilation genes mainly showed high expression levels at the early berry development stage (flowering and first berry expansion stages), in which VvAS showed higher expression levels at FE-1 and FE-2, VvGS showed higher expression levels at FE-3 and FE-4, VvGDH showed higher expression levels at FE-4 and FE-5, and VvNR and VvNIR showed the highest expression peaks at FE-3 and FE-7 respectively (Fig. 1). By contrast, the four P uptake genes mainly exhibited high expression levels at the late berry developmental stages, for example, VvSIRK showed higher expression levels at FE-7 and FE-8, VvPAP showed higher expression levels at FE-3 and FE-4, and VvPHO1 showed higher expression levels at FE-7 (Fig. 2). Similarly, the four K uptake genes also showed high expression levels at the late berry developmental stages, for example, VvSIRK showed higher expression levels at FE-3 and FE-4; VvKUPI showed higher expression levels at FE-7, FE-8, and FE-9; VvSORK showed higher expression levels at FE-7 and FE-8; and VvKUP2 showed higher expression levels at FE-4 and FE-8 (Fig. 3).

Verifying the Feasibility of Gene Expression-Based Fertilization Strategy by Testing the Responses of the N–P–K Uptake and Metabolism Genes to Fertilization

As the expression levels of N–P–K uptake and metabolism genes reflect the absorption and transportation effectiveness of N, P, and K elements, by testing the expression levels of N–P–K uptake and metabolism genes in the fertilized grapevines and the controls during the flowering and berry developmental periods, we can verify whether the fertilizers were more efficiently absorbed and transported during the particular stages when the N–P–K uptake and metabolism genes were highly expressed. Generally, our results showed that at the specific stages where N–P–K uptake and metabolism genes were highly expressed, these genes also showed more active responses to fertilization. For example, as mentioned above, N assimilation genes VvGDH, VvNR, VvNIR, VvAS, and VvGS showed high expression levels at the FE-4, FE-3,
After fertilization treatment, these genes in the fertilized grapevine also showed higher expression levels in the above stages compared to the control, in which VvGDH, VvNR, and VvGS exhibited higher expression levels at FE-3 and FE-4; however, VvNIR showed higher expression levels at FE-7 and VvAS showed higher expression levels at FE-1, FE-2, FE-3, and FE-4 (Fig. 4). The same phenomenon
was also observed in the P and K uptake genes. After the fertilization treatment, among the P uptake genes, \( VvPHT1–4 \) showed higher expression levels at FE-5, FE-9, and FE-10; \( VvPHT2–1 \) showed higher expression levels at FE-1, FE-2, FE-8, and FE-9; and \( VvPAP \) showed higher expression levels at FE-1, FE-2, FE-3, and FE-5 (Fig. 5). Among the K uptake genes, \( VvSIRK \) showed higher expression levels at FE-4; \( VvKUP1 \) showed higher expression levels at FE-5, FE-8, FE-9, and FE-10; \( VvSORK \) showed higher expression levels at FE-8, FE-9, and FE-10; and \( VvKUP2 \) showed higher expression levels at FE-4, FE-7, and FE-8 (Fig. 6). All these genes showed more positive responses to fertilization than their corresponding controls under fertilization treatment at the stages in which high expression levels were detected in the previous study (Fig. 2 and Fig. 3).

This analysis suggested that at the specific stages where the N–P–K uptake and metabolism genes were highly expressed, grapevine also responded more positively to fertilization, which indicates that grapevine has higher nutrient absorption, transportation, and assimilation efficiency at these stages, so applying fertilizers at these stages can better enhance the growth and development of grapevine. It is therefore feasible to predict grapevine's nutritional requirements according to the expression information of N–P–K uptake and metabolism genes and then applying fertilizers following these guidelines.

Verifying the Workability of Gene Expression-Based Fertilization Strategy by Grape Quality Investigation

Expression analysis of the N–P–K uptake and metabolism genes in the fertilized grapevines and controls has shown that applying fertilizers at the stages where N–P–K uptake and metabolism genes were highly expressed made grapevine respond to fertilization more positively; theoretically, grape quality will also be more greatly improved. To verify this hypothesis, and validate the workability of gene expression-based fertilization strategies, grape quality traits, including single berry weight, cluster weight, soluble solid content, and total sugar content under fertilization treatments were investigated. These results showed that applying fertilizers at the first and second berry expansion stage, and the veraison stage can improve the quality of grape much more than fertilizer application at any other stages. For example, when fertilizers were applied at the first berry expansion stage (FE-3 and FE-4), single berry weight, cluster weight, and soluble solid content all increased greatly compared to the control (Fig. 7a–c); when fertilizers were applied at the second berry expansion stage (FE-7 and FE-8), all four grape quality traits increased remarkably compared to the control (Fig. 7a–d); when fertilizers were applied at the veraison stage (FE-9 and FE-10), both soluble solid content and total sugar content increased significantly compared to the control (Fig. 7c,d). By contrast, when fertilizers were applied at the seed stone hardening stage (FE-5 and FE-6), grape quality had almost no change compared to the control and – even worse – grape total sugar content decreased slightly compared to the control (Fig. 7d). Consistently, most of the N–P–K uptake and metabolism genes showed higher expression levels at these three stages, in which \( VvSIRK \), \( VvKUP2 \), \( VvGDH \), \( VvNR \), \( VvGS \), and \( VvPAP \) were highly expressed at the first berry expansion stage; \( VvNIR \), \( VvPHT2–1 \), \( VvPHO1 \), \( VvKUP1 \), \( VvKUP2 \), and \( VvSORK \) were highly expressed at the second berry expansion stage; and \( VvPHT1–4 \) and \( VvKUP1 \) were highly expressed at the veraison stage (Fig. 1, Fig. 2, Fig. 3). In addition,
Fig. 4. Comparison of expression levels of N metabolism genes between fertilized grapevine and the control. Fertilizer treatments 1 to 10 (FE-1 to FE-10) denote 10 fertilization stages; 1 to 3 denote three sampling times every 3 d after fertilization. Each reaction was repeated three times and the template amount was expressed relative to Actin. Grapevine samples were derived from the Summer Black cultivar. The expression levels of the fertilizer-treated samples are expressed as a ratio relative to their corresponding controls, which were set to 1 and are shown in the column labeled CK. *, Significant difference at the 0.05 level; **, significant difference at the 0.01 level; ***, significant difference at the 0.001 level.
Fig. 5. Comparison of expression levels of P uptake genes between the fertilized grapevine and the control. Fertilizer treatments 1 to 10 (FE-1 to FE-10) denote 10 fertilization stages; 1 to 3 denote three sampling times every 3 d after fertilization. Each reaction was repeated three times and the template amount was expressed relative to Actin. Grapevine samples were derived from the ‘Summer Black’ cultivar. The expression levels of the fertilizer-treated samples are expressed as a ratio relative to their corresponding controls, which were set to 1 and are shown in the column labeled CK. *, significant difference at the 0.05 level; **, significant difference at the 0.01 level; ***, significant difference at the 0.001 level.
Fig. 6. Comparison of the expression levels of K uptake genes between the fertilized grapevine and the control. Fertilizer treatments 1 to 10 (FE-1 to FE-10) denote 10 fertilization stages; 1 to 3 denote three sampling times every 3 d after fertilization. Each reaction was repeated three times and the template amount was expressed relative to Actin. Grapevine samples were derived from the Summer Black cultivar. The expression levels of the fertilizer-treated samples are expressed as a ratio relative to their corresponding controls, which were set to 1 and are shown in the column labeled CK. *, significant difference at the 0.05 level; **, significant difference at the 0.01 level; ***, significant difference at the 0.001 level.
N–P–K uptake and metabolism genes in these three stages were also more positively influenced by fertilization, as described earlier. All these findings suggest that applying fertilizers on the basis of the expression information of N–P–K uptake and metabolism genes is a feasible and practical fertilization strategy that can be used in the production of grape or even other crops.

Predicting Grapevine’s Nutritional Requirements Based on N–P–K Uptake and Metabolism Gene Expression Levels

Nitrogen–phosphate–potassium uptake and metabolism gene expression levels have been verified as being feasible to predict the nutritional requirements of grapevine during the development period. Based on our findings, we made a diagram to depict the dynamic changes in grapevine’s nutritional requirements during the flowering and berry developmental periods (Fig. 8). This diagram is depicted in terms of gene expression levels, which is more accurate and science-based than traditional fertilizer application techniques. These findings can assist in higher grape production and also act as guidelines for farmers in future.

At the flowering stage, three P uptake genes \textit{VvPHT2–1}, \textit{VvPHO1}, and \textit{VvPAP} were highly expressed after applying fertilizers. Their expression levels increased over time in FE-1; however, in FE-2, their expression levels increased first and then declined. This indicated that P nutrients are greatly needed by grapevine during the flowering stage and P fertilizers will be more effective in the early flowering stage than that in the later stages (Fig. 5). At the first berry expansion stage, the N assimilation genes \textit{VvGDH}, \textit{VvNR}, \textit{VvGS} and \textit{VvAS} and the K uptake genes \textit{VvSIRK} and \textit{VvKUP2} were all highly expressed after fertilization treatment. Moreover, the expression of the four N assimilation genes first increased and then decreased, whereas the expression of the two K uptake genes kept increasing after the application of fertilizers (Fig. 4, Fig. 6). This suggests that grapevine has higher requirements for N and K nutrients at this stage, and N fertilizers have longer effectiveness than K fertilizers. At the seed stone hardening stage, only the P uptake gene \textit{VvPHT1–4} and the K uptake gene \textit{VvKUP1} were highly expressed compared to the control (Fig. 5 and Fig. 6), which suggests that grapevine needs less nutrient at this stage. At the second berry expansion stage, the P uptake genes \textit{VvPHT2–1} and \textit{VvPHO1} and the K uptake genes \textit{VvKUP1}, \textit{VvKUP2}, and \textit{VvSORK} showed higher expression levels after fertilization. In addition, the expression of the two phosphate uptake genes kept increasing over time after fertilization but the expression of K uptake genes first increased and then decreased (Fig. 5, Fig. 6), which indicates that grapevine has more need for P and K nutrients in this particular stage and that the effectiveness of P fertilizers could be more durable than that of K fertilizers. At the veraison stage, the P uptake genes \textit{VvPHT2–1}, \textit{VvPHT1–4}, and \textit{VvPHO1} and the K uptake
genes VvKUP1 and VvSORK were highly expressed after fertilization treatment. However, their expression levels all showed declining trends subsequently, which suggests that grapevine has a higher need for P and K fertilizers at this stage, although these fertilizers have relatively short effectiveness (Fig. 5, Fig. 6).

**Discussion**

Nowadays, most of the research on crop fertilization technologies is mainly focused on plant nutrition diagnosis, soil testing and formulation of fertilizers (Bhargava and Sumner, 1987; Li et al., 2007; Marangoni et al., 2001; Romero et al., 2010; Wheeler and Pickering, 2003). These techniques enable us to learn about and monitor the nutritional status of soil, nutrient uptake, and metabolism levels in plants during growth, thus providing us with certain guidelines for fertilizer application. However, it is not possible to predict the specific nutritional requirements of different developmental stages. What is worse, these diagnostic techniques could be influenced by many internal and external factors and are not sensitive enough to be used as fertilization guidelines. However, by monitoring the expression levels of N–P–K uptake and metabolism genes, we can figure out the specific nutrient requirements of different developmental stages and also predict the time when fertilizers can be absorbed more efficiently, thus enabling us to apply fertilizers with the right nutrient type at the perfect time. Recent advancements in molecular biotechnology, especially the development of one-step PCR, which is a simple, rapid, and sensitive technique for detecting gene expression levels, makes it possible to monitor the gene expression status of the crops in real time. In this case, by monitoring the expression of N–P–K uptake and metabolism genes in the crops, we can figure out the actual picture of the absorption, transportation, and metabolic status of N, P, and K during the development of crops. With this information, we can predict the nutritional requirements of crops and then apply fertilizers timely and appropriately and at the right time. In this research, we validated the feasibility and workability of gene expression-based fertilization strategies. During the flower and berry development period, fertilizers could be more efficiently absorbed and transported by grapevine and ultimately resulted in better quality and yield when they were applied at the stages when the N–P–K uptake and metabolism genes were highly expressed. With ongoing in-depth research in molecular biology, the genome sequences of many plant species have been made available and more and more genes are being cloned, meaning that not only nutrient uptake and metabolism genes but also lots of stress-related genes such as plant diseases, drought, salinity, and cold stress tolerance genes in different plants and cultivars will be discovered, from which expression information can be derived and used to predict the damage caused by stress and then take preventive measures. This work possibly opens a new era of applying gene information to guide agricultural production. As predicted by Boss and Thomas (2000), gene information and engineering is being applied in many aspects of grape production, meaning that the era of applying gene information in practical fruit crop production has arrived.

Grapevines are perennial plants with long lifecycles (He, 1998; Kong, 2004) and there are key stages in its phenology that have a greater demand for nutrients than others. Applying fertilizers at different developmental stages of grapevine can make a big difference in grape quality and yield (Peacock et al., 1991). Christensen et al. (1994) and Conradie (2005) reported that N is most
critically needed by grapevines during the period of rapid shoot growth in the spring through to blooming and early berry development. This need declines as grapes ripen. Application of N fertilizers during this period can greatly stimulate shoot growth and enhance fruit set. Niu et al. (2008) reported that K should be sufficiently and promptly applied at both the full bloom and fruit coloring stages to table grape cultivars to ensure fruit growth as well as high K use efficiency of the fertilizer. Ma et al. (2014) reported that applying P fertilizers at the rapid shoot growth stage and the fruit expansion stage can improve the utilization ratio of P fertilizer and better meet the P requirements of ‘Moldova’ grapes.

In the present work, the nutritional requirements of grapevine during the flowering and berry developmental periods were depicted in terms of gene expression levels and a grapevine nutritional requirements diagram was constructed (Fig. 8), in which grapevine requires more P nutrients at the flowering stage, more N nutrients at the first berry expansion stage, fewer nutrients at the seed stone hardening stage, and more P and K nutrients at the second berry expansion stage and the veraison stage. Nitrogen is only greatly needed by grapevine at the earlier stages of grape development, which is consistent with the previous reports. However, excessive N fertilizer application at the early growth periods of grapevine may also lead to negative consequences, such as excessive vegetative growth, reduced fruit quality, or delayed ripening after the fruit sets (Bell and Henschke, 2005; Keller, 2005; Neilsen et al., 2010). Phosphate and K are major nutrients involved in the synthesis and translation of organic materials in grape berries (Spayd et al., 1993; Xie et al., 2005) primarily needed at the later developmental stages of grape berry (second berry expansion stage and veraison stage), possibly because of the drastic biosynthetic and metabolic activities of organic materials such as sugars, organic acids, and polyphenols in the grape berries during these stages. Although we have figured out the specific nutritional requirements during the flowering and grape berry developmental periods, there are many other important developmental stages in grapevine phenology that are critical to the growth and development of grape, such as the flower bud differentiation and early shoot growth stages. Figuring out grapevine’s requirements for nutrients at these stages is also crucial for optimizing the quality and yield of grape, which needs to be studied in future.

In this study, urea (50 g plant) and KH₂PO₄ (100 g per plant) were applied to investigate the responses of N–P–K uptake and metabolism genes to fertilization. These two fertilizers are the most widely used in agricultural crop production. However, N, P, and K fertilizers come in many types and each type has unique characteristics that may suit a specific crop species. In addition, fertilizer doses and combinations of different fertilizers also play important roles in optimizing the yield and quality of grape (Dai et al., 2013; Delgado et al., 2004; Zhang et al., 2010b). Although applying fertilizers on the basis of gene expression has been verified by our findings as being a feasible and practical fertilization strategy, further evaluation of the effects of different fertilizer types, combinations, and application rates according to gene expression information will allow us to achieve a broader goal, which is to predict the nutritional requirements of plants and then applying fertilizers with the optimal type at the most reasonable application rate and with the best combination of fertilizers.

Conclusions
The gene expression-based fertilization strategy is an effective and applicable technique. The nutritional requirements of grapevine during the flowering and berry developmental periods were summarized in terms of gene expression levels, in which grapevine needs more P at the flowering stage, more N at the first berry expansion stage, less nutrient at the seed stone hardening stage, and more P and K at the second berry expansion stage and veraison stage. This study will contribute to improving traditional fertilization techniques and also is one of the novel and initial findings regarding the application of fertilizer in vineyards for better cultivation of grapevine during common cultural practices.

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