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# Proteomic analysis of sex conversion induced by CPPU in male grapevine of *Vitis amurensis*

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## Summary

**If N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) could induce sex conversion in male plants of *Vitis amurensis* Rupr., this would reduce blindness of selection for male parents according to the fruit characters in cold-tolerant and disease-tolerant grape crossbreeding. Flower bud samples of male plants were treated with 100 mg·L$^{-1}$ CPPU at 15 days before anthesis. Two-dimensional gel electrophoresis (2-DE) was used to analyze the proteins related to sex conversion at different development time points. More than 600 protein spots were detected. Among them, 31 differentially expressed proteins were identified by MALDI-TOF/TOF, and 24 protein spots could be assigned to a probable function. Seventeen proteins participated in the sex conversion and with complex interaction. Sex conversion might receive the ROS signal in the beginning, and then pollen tube proteins were proposed to down-regulate to repress the stamen development, while the up-regulated cell elongation protein might promote the development of pistil. Adenine phosphoribosyl transferase 3 was proposed as the key protein in the sex organ conversion that was up-regulated by CPPU in the male *V. amurensis* achieving the ability to fruit in the end.**

K e y w o r d s : *Vitis amurensis* Rupr, male grapevine, sex conversion, CPPU, proteome.

## Introduction

*Vitis amurensis* Rupr. (Vitaceae) is a wild grapevine species originating from East Asia (Blasi *et al*. 2011) and is mainly distributed in Korea, Japan, and China (Jang *et al*. 2007).

Its fruit is used to make juice and wine, while the roots and stems have anti-oxidant and anti-inflammatory activities (Lee *et al*. 2004, Huang and Lin 2012). Moreover, *V. amurensis* is one of the most significant strains in grapevine breeding due to its resistance to low temperature and disease. As one of the most cold-tolerant grapes, its stem can endure -40 to -50 °C (Ma *et al*. 2010). *V. amurensis* also exhibits resistance to powdery mildew (Wan *et al*. 2007), anthracnose and white rot (Li *et al*. 2008). These favorable characteristics in *V. amurensis* have prompted grapevine breeders to include it in their breeding programs. However, male *V. amurensis* cannot fruit, which makes it blind for the selection of *V. amurensis* male parents in grape crossbreeding.

Systematic attempts were started in 1965 to induce sex conversion in male vines of *Vitis*. Two reports on sex conversion in a male *Vitis vinifera* L. and other *Vitis* species induced by kinin were found in 1966 and 1971 (Negi and Olmo 1966, 1971). For chlorfenuron (CPPU) is the most effective synthetic cytokinin and is effective in enlarging fruit (Iwahori *et al*. 1988), controlling the fruit set and fruit growth (Sugiyama and Yamaki 1995), changing flower morphology (Takaaki and Koji. 2006) and inducing parthenocarpy of female flowers (Yu 1999). Recently, CPPU was also found to be effective in induction of sex conversion in male *Vitis amurensis*. Flower buds of male *V. amurensis* treated by CPPU about 20 d before anthesis alters the sex from male to hermaphrodite and development of fruit (Ai *et al*. 2002), which is valuable to grapevine breeding.

Previous studies correlated to sex determination have demonstrated that the flower sex is determined by genes and regulated by hormones (Heslop-Harrison 1957, Deputy *et al*. 2002). The genes controlling flower sex in grapevines have already been proposed in several genetic mapping efforts based on molecular markers (Fechter *et al*. 2012). How hormones contribute to flower development as the principal transducers of genetic information was revealed (Chandler 2011), while the molecular and physiological mechanisms underlying *Vitis* sex conversion are still unknown. In the last decade, proteomics has become an important approach to the understanding of the molecular physiology of plant development and has been extensively used to identify and characterize proteins involved in plant development (Takac *et al*. 2011) or responses to hormones (Zhang *et al*. 2012). With the completion of grape genome sequencing (Jaillon *et al* 2007), it is possible to analyze the sex conversion of male *V. amurensis* using the proteome. In this study, proteomics was applied in order to obtain a more detailed understanding of the mechanisms of male *V. amurensis* sex conversion induced by CPPU. Two-DE followed by MALDI-TOF/TOF were used to investigate and analyze the biological function of the differentially expressed proteins in male *V. amurensis* flower buds induced by CPPU at different flower development time points, and also to provide a dynamic picture of the sex conversion proteome. This study offers new insights to the physiological mechanism of sex conversion in male *V. amurensis* flower buds and provides theoretical evidence for better regulation of *V. amurensis* sex and quality of fruit.

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## Material and Methods

Plant materials and CPPU treatment: Male *V. amurensis* cultivar 73077 was grown at the orchard of the National Field Gene Bank for Amur Grapevine (Zuojia, China). The vines were divided into two groups: CPPU treated group and control group. Each group consisted of 8 replicate plants. The flower clusters of the CPPU treated group were dipped in 100 $mg \cdot L^{-1}$ CPPU-ethanol solution for 3 s on May 25, 2011, approximately 15 d before anthesis. Water instead of CPPU was used for the control group. Flower bud samples were taken at eight flower development time points (0.5, 1, 2, 3, 4, 5, 6, 8 d ) after treatment from the CPPU treated group (CT 0.5, 1, 2, 3, 4, 5, 6, 8) and control group (CK 0.5, 1, 2, 3, 4, 5, 6, 8). Collected samples were immediately kept in liquid nitrogen, and stored at -80 °C for further analysis.

Protein extraction and quantification: Protein extraction was performed using the phenol protocol (Faurobert *et al*. 2007) with some modifications. Flower buds (1.5 g) were finely powdered with 0.15 g PVPP in liquid nitrogen and then be suspended in 15 mL ice-cold extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl, 0.05 M EDTA, 0.5 M ascorbic acid, 2 % β-mercaptoethanol and 1 % PMSF; pH 8.0) in a 50-mL centrifuge tube, vortexed, and incubated by shaking for 10 min on ice. Afterward, each sample was mixed with an equal volume of Tris-saturated phenol (pH 8.0), and the solutions were incubated on a shaker for 30 min on ice. The upper phenol extraction was obtained by centrifugation for 20 min at 10,000 g and 4 °C. An equal volume of extraction buffer was added to the upper phenol extraction, the sample was vortexed and centrifuged again, and this procedure was repeated three times. The final collection of phenol was precipitated overnight with five volumes of 0.1 M ammonium acetate/methanol at -20 °C. After centrifugation for 20 min at 10,000 g and 4 °C, the pellets were washed four times with cooled methanol and finally with cooled acetone. The pellets were then dried at -20 °C.

The dried powder was dissolved at room temperature in lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 65 mM DTT and 0.2 % Bio-lyte), vortexed for 10 min, and then centrifuged for 10 min at 13,000 rpm. The supernatants were collected and the protein content was determined according to the procedure of Bradford (Richard 2003).

2-dimensional electrophoresis: 2-dimensional electrophoresis (2-DE) was performed using the Protean IEF and Protean II XL systems (Bio-Rad, USA). A total of 900 μg protein samples were diluted with rehydration buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 65 mM DTT and 0.2 % Bio-lyte and 0.002 % Bromophenol Blue) to a final volume of 300 μL and loaded onto a 17 cm, pH 4-7 non-linear IPG strips (3-10 NL Ready strips, Bio-Rad, USA). After rehydration, IEF was performed at 20 °C under the following conditions: 250V for 30 min, 1000V for 1 h, 10,000V for 5 h, and then run at 10,000 V for 70,000 Vh with 50 mA per strip. Following IEF, the strips were equilibrated for 15 min [6 M urea, 20 % (v/v) glycerol, 2 % (w/v) sodium dodecyl sulfate, 0.375 M Tris-HCl (pH 8.8) and 2 % (w/v) DTT] followed by 15 min equilibration in the same buffer containing 2.5 % (w/v) iodoacetamide without DTT with gentle shaking. Second dimension electrophoresis was carried out on 12 % SDS polyacrylamide gels, and staining with CBB G-250.

Gel scanning and analysis: 2-DE gels were scanned with image scanner UMAX Power Look 2100 XL and analyzed with the PDQuest 8.0 2D Analysis Software (Bio-Rad, USA). Spots were detected automatically by the Spot Detection Parameter Wizard using the Gaussian model and streak removal setting and normalized using local regression model to compare spot quantities across gels accurately. Only spots statistically significant in Student's t-test at a level of 95 %, with a fold change of ± 2.0 and present only in CPPU treatment or in control gels were accepted as differentially expressed and selected for mass spectrometry analysis.

Protein in-gel tryptic digestion: The differentially expressed protein spots were manually excised from the 2-DE gels and placed into a 96-well microtitre plate and destained with a solution of 25 mM ammonium bicarbonate in 50 % ACN for 30 min at 37 °C. Gel pieces were then washed twice with deionized water and shrunk by dehydration in ACN followed by incubation in a digestion buffer containing 25 mM ammonium bicarbonate and 12.5 $ng \cdot \mu L^{-1}$ trypsin for 30 min at 4 °C. After the gels were digested more than 12 h at 37 °C, the resulting peptides were extracted twice using 0.1 % TFA in 50 % ACN and evaporated to dryness in $N_2$. Finally, the samples were redissolved in ACN/water 1:1 and 1 % acetic acid for MALDI-TOF/TOF MS analysis.

Mass spectrum and database search: The redissolved samples were subjected to matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/TOF) analysis using a 5800 TOF/TOF (AB Sciex, USA) at Proteome Center, Fudan University (Shanghai, China). The UV laser was operated at a 200 Hz repetition rate with wavelength of 355 nm. The accelerated voltage was operated at 20 kV, and mass resolution was maximized at 1,500 Da. The mass range was scanned from 700 to 3,600 Da. Myoglobin digested by trypsin was used to calibrate the mass instrument with internal calibration mode. All the spectra of the real samples were acquired using a default mode.

The acquired data were automatically submitted to the NCBInr database for identification by using GPS Explorer software (Applied Biosystem, USA). The following parameters were used in the search: plants, protein molecular mass ranged from 700 to 3,600 Da, trypsin digest with one missing cleavage, peptide tolerance of 100 ppm MS/MS tolerance of 0.6 Da and possible oxidation of methionine. Searches were performed in the full range of Mr and pI of the target proteins.

## Results

Inflorescences of the CPPU treated male *V. amurensis* and two-dimensional electrophoresis analysis: Forchlorfenuron (CPPU) induced the sex conversion of male *Vitis amuren-*

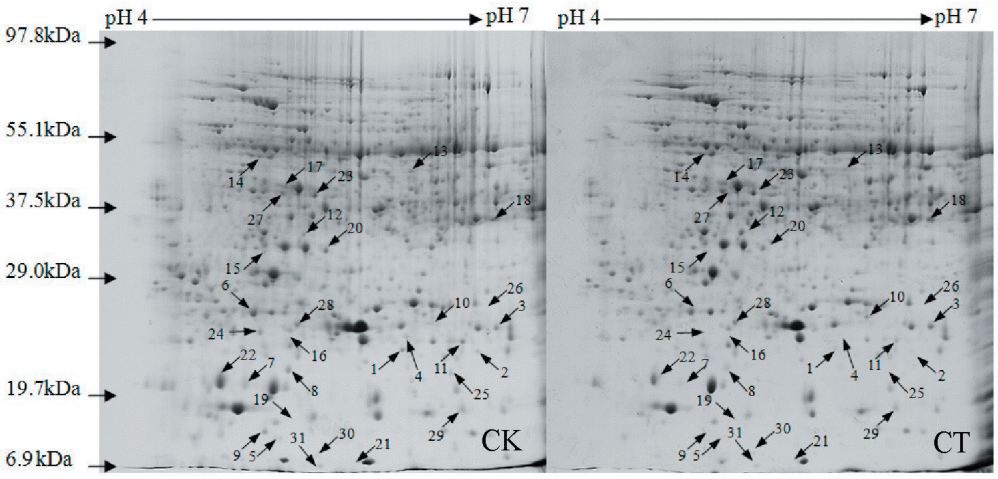


Fig. 1: 2-DE gel samples of male *V. amurensis* flower buds treated by CPPU at the forth day. Relative molecular weights of proteins are shown on the left. 2-DE was performed using 900 μg of protein, linear 17 cm IPG strips (pH 4-7) and 12 % SDS-PAGE gels for second dimension electrophoresis. Gels were stained with CCB G250.

*sis* Rupr which resulted in fruit and seed production in the autumn. A comparative study of the soluble flower bud proteome between control and CPPU treatment at different development time points was performed, aimed to identify and characterize proteins involved in sex conversion of the male *V. amurensis*. The 2-DE gels are shown in Fig. 1. About 600 protein spots were detected by PDQuest 8.0.1 software on each gel. CPPU induced flower bud proteome change at each development time point, with a total of 31 proteins (CK4 and CT4 were as example shown in Fig. 1) were found between control and CPPU treatment.

Identification and functional classification of differentially expressed proteins: The differentially expressed protein spots (31) were digested using trypsin and subjected to MALDI-TOF/TOF, and 24 proteins were successfully identified with probability ($p < 0.05$ %) according to NCBInr database (Table). The identified proteins were found to be involved in different metabolic pathways and processes, the functional categories (Fig. 2) include antioxidant and defense (spot 2, 4, 9, 11, 13, 18 and 21), energy balance (spot 6, 8, 27), pollen tube growth (spot 1, 14, 15, 16, 17), cell elongation (spot 23), flower sex of *Vitis* (spot 10), and unclassified proteins (spot 3, 5, 7, 12, 20, 22, 24). The remaining unidentified proteins are the spots 19, 25, 26, 28, 29, 30 and 31.

Spots changed by CPPU treatment: Magnified views of the 31 protein spots are highlighted in Fig. 3. We found that great changes have occurred between the CT and CK treatments. Compared with the CK, there were 12 spots up-regulated treated by CPPU, for example, spot 10 (APT) is expressed higher than CK at the whole process and significantly higher at 3, 4, 5, 8 d; spot 13 (Chalcone synthase) and spot 12 were up-regulated at two treated days; spot 6 (Chloroplast chlorophyll a/b binding protein), 21 (Glycine-rich RNA-binding protein), 23 (Ribosome-associated protein p40), 27 (Succinyl-CoA synthetase beta chain), 7, 28, 19, 30 and 31 were changed at one time point. CPPU induced 19 down-regulated spots, including spot 1 (Proteasome subunit beta type-2-A) changed at the early stages (0.5, 1, 2, 3 d); spot 4 (Mn-SOD), 9 (Cu/Zn-SOD) and 24 were down-regulated at two treated days; spot 2 (Glycine-rich RNA-binding protein), 8 (ATP synthase Dchain), 11 (Mn-SOD), 14 (Tubulin), 15 (Alpha-soluble NSF attachment protein), 16 (PBC1), 17 (Actin), 18 (Polyphenol oxidase), 3, 5, 20, 22, 25, 26 and 29 were changed at one time point.

## Discussion

Comparison of control and CPPU treated flower buds at different development time points revealed that their proteomes are possibly regulated by hormone levels and by the developmental stages, which resulted in differential expression of proteins involved in metabolic pathways and flower sex. Because there were some proteins contributing to two or more pathways with complex functions, we classified the 17 proteins mainly according to the relation to flower development and hormone regulation and the key proteins regulated in the sex conversion were discussed as below.

Proteins related to antioxidant and defense as the signal in sex conversion: Accumulation of reactive oxygen species (ROS) can damage plant tissue under biotic or environmental stress (Asada 1987, Xu *et al*. 2006). Superoxide dismutases (SODs) act as the first line of defense against ROS by dismutating $O_2^-$ to $H_2O_2$ (McCord and Fridovich 1969), and have the important role to detoxify ROS and redox balance (Kliebenstein *et al*. 1998). Three classes of SODs have been defined based on their metal cofactors: Cu/Zn SOD, Mn-SOD and Fe-SOD (Fridovich 1975). Results showed that three proteins related to ROS scavenging mechanisms were identified as Cu/Zn-SOD (spot 9) and Mn-SOD (spot 4 and 11). Three spots were all down-regulated in sex conversion process indicating that CPPU exerts a negative effect on the ROS-scavenging mechanisms. Meanwhile, several studies have revealed that ROS play a role in signal transduction (Apostol *et al*. 1989). Examples of how changes in the concentrations of ROS are perceived and transferred into signals that change the transcription of genes has been

Table

Identification of differential proteins of control and CPPU treated male *V. amurensis* flower buds with MALDI-TOF/TOF and database searching

| Spot No. | Protein Name | Species | Accession No. | Theoretical Mr (kDa)/pI | Observed Mr (kDa)/pI | Pep. Count | Protein Score | Total Ion Score |
|---|---|---|---|---|---|---|---|---|
| Antioxidant and defense | | | | | | | | |
| 4 | Superoxide dismutase (manganese) | *Hevea brasiliensis* | gi\|348137 | 25.8/7.1 | 23.8/6.1 | 6 | 203 | 167 |
| 9 | Cu/Zn superoxide dismutase | *Vitis vinifera* | gi\|3063643 | 21.7/5.87 | 14.37/5.2 | 5 | 421 | 384 |
| 11 | Manganese superoxide dismutase | *Vitis vinifera* | gi\|161778782 | 25.3/6.79 | 23.2/6.4 | 10 | 318 | 248 |
| 13 | Chalcone synthase | *Vitis vinifera* | gi\|18376655 | 42. 9/6.1 | 49.0/6.1 | 10 | 172 | 138 |
| 18 | Polyphenol oxidase | *Vitis vinifera* | gi\|1785613 | 67.3/6.39 | 39.3/6.7 | 12 | 315 | 271 |
| 2 | Glycine rich protein 2 | *Arabidopsis thaliana* | gi\|15234010 | 19.1/5.62 | 22.1/6.5 | 2 | 166 | 156 |
| 21 | Glycine-rich RNA-binding protein | *Oryza sativa* Japonica Group | gi\|50881454 | 15.9/6.29 | 10.8/5.8 | 5 | 131 | 98 |
| Energy metabolism | | | | | | | | |
| 6 | Putative chloroplast chlorophyll a/b binding protein | *Vitis* hybrid cultivar | gi\|163914163 | 24.9/5.26 | 27.55/5.2 | 6 | 160 | 131 |
| 8 | ATP synthase Dchain, mitochondrial | *Ricinus communis* | gi\|223530804 | 19.7/5.33 | 20.41/5.4 | 13 | 186 | 110 |
| 27 | Succinyl-CoA synthetase beta chain | *Ricinus communis* | gi\|223530658 | 45.4/5.86 | 43.0/5.4 | 12 | 212 | 174 |
| Pollen tube growth | | | | | | | | |
| 1 | Proteasome subunit beta type-2-A | *Arabidopsis thaliana* | gi\|17380179 | 22.5/5.95 | 22.5/6.0 | 8 | 226 | 179 |
| 16 | PBC1 (20S proteasome beta subunit C1); peptidase isoform 2 | *Vitis vinifera* | gi\|225459611 | 19.7/5.69 | 27.55/5.4 | 4 | 231 | 205 |
| 14 | Alpha-tubulin | *Gossypium hirsutum* | gi\|134035504 | 49.5/4.93 | 51.9/5.2 | 11 | 555 | 479 |
| 17 | Actin | *Mesostigma viride* | gi\|3127135 | 41.6/5.3 | 44.2/5.4 | 9 | 324 | 284 |
| 15 | Alpha-soluble NSF attachment protein | *Ricinus communis* | gi\|223539951 | 32.5/5.12 | 32.9/5.2 | 7 | 96 | 70 |
| Cell elongation | | | | | | | | |
| 23 | Ribosome-associated protein p40 | *Glycine max* | gi\|2444420 | 33.9/5.1 | 43.9/5.6 | 9 | 375 | 331 |
| Flower sex of Vitis | | | | | | | | |
| 10 | Adenine phosphoribosyl transferase 3 | *Arabidopsis thaliana* | gi\|15235709 | 20.3/5.83 | 24.6/6.2 | 4 | 233 | 217 |
| Unclassified | | | | | | | | |
| 3 | Hypothetical protein | *Vitis vinifera* | gi\|225436699 | 24.6/6.17 | 23.6/6.7 | 9 | 320 | 262 |
| 5 | Hypothetical protein isoform 1 | *Vitis vinifera* | gi\|225451122 | 15.3/5.49 | 13.6/5.3 | 4 | 291 | 257 |
| 7 | Hypothetical protein VITISV_008439 | *Vitis vinifera* | gi\|147776917 | 17.2/5.15 | 19.8/5.1 | 10 | 302 | 234 |
| 12 | Hypothetical protein VITISV_003812 | *Vitis vinifera* | gi\|147843260 | 34.3 / 5.23 | 45.03/5.5 | 8 | 517 | 472 |
| 20 | Hypothetical protein | *Vitis vinifera* | gi\|225430273 | 34.4/5.54 | 33.8/5.6 | 15 | 547 | 423 |
| 22 | Hypothetical protein VITISV_008439 | *Vitis vinifera* | gi\|147776917 | 17.2/5.15 | 20.5/5.0 | 10 | 323 | 253 |
| 24 | Hypothetical protein | *Vitis vinifera* | gi\|225466111 | 23.7/5.64 | 24.1/5.2 | 12 | 464 | 367 |
| Unidentified | | | | | | | | |
| 19 | Hypothetical protein VITISV_003812 | *Vitis vinifera* | gi\|147843260 | 34.27/5.23 | 15.6/5.4 | 8 | 70 | - |
| 25 | Rab2/RabB-family small GTPase | *Physcomitrella patens* | gi\|162671515 | 23.3/6.96 | 20.36/6.51 | 8 | 68 | - |
| 26 | NADH dehydrogenase subunit | *Aethionema grandiflorum* | gi\|139389319 | 19.51/7.53 | 24.8/6.6 | 5 | 44 | - |
| 28 | Chaperonin | *Zea mays* | gi\|195637206 | 25.74/8.8 | 24.4/5.5 | 6 | 66 | - |
| 29 | Unknown | *Zea mays* | gi\|194698300 | 28.02/8.94 | 15.6/6.4 | 8 | 61 | |
| 30 | Conserved hypothetical protein | *Ricinus communis* | gi\|223536260 | 14.55/5.27 | 11.8/5.6 | 5 | 74 | - |
| 31 | Hypothetical protein | *Vitis vinifera* | gi\|225445883 | 14.77/5.83 | 10.3/5.6 | 3 | 67 | - |

Note: Protein scores greater than 75 are significant ($p < 0.05$).

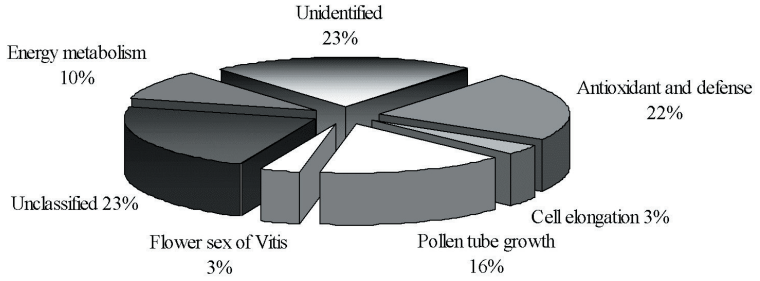


Fig. 2: Pie chart showing the distribution of the proteins identified under CPPU treatment according to their functions. Seven protein groups were categorized based on the relations to sex conversion.

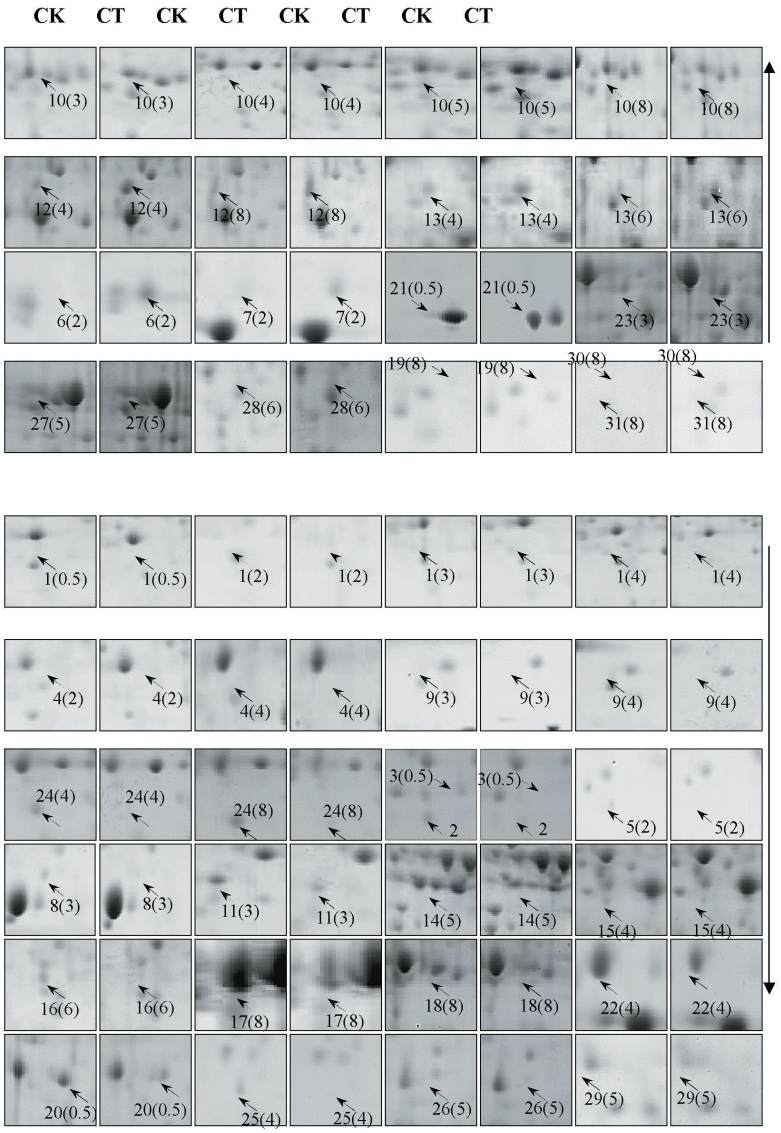


Fig. 3: Partial magnifications of differential proteins on 2-DE gels of control (CK) and CPPU treated (CT) male *V. amurensis* flower buds at different flower development time points. The above 12 proteins were up-regulated after CPPU treatment and the below 19 proteins were down-regulated. Numbers in gels represent Spot No. and the corresponding treat days shown in brackets.

published (Laloi *et al*. 2004). In the present study, ROS are emerging as important regulators of plant development mainly because of their signaling role (Gapper and Dolan 2006, Foreman *et al.* 2003) and ROS signaling requires regulation of ROS-scavenging mechanisms (Kawk *et al*. 2006). Therefore, we could confer that ROS act as signal molecules so as to promote sex conversion, and CPPU controls ROS signal by regulating ROS-scavenging mechanisms. Other defense proteins were also detected in the sex conversion, such as flavonoid related protein: chalcone synthase (spot 13) and PPO (spot 18), glycine rich RNA-binding protein (spot 2 and 21). All of these may have a

relation to pollen development, while the mechanisms of their involvement in sex conversion of male *V. amurensis* are still unclear.

Proteins related to energy metabolism to balanced energy in sex conversion: Three energy proteins were detected in sex conversion of male *V. amurensis*. ATP synthase in chloroplast and mitochondria are important enzyme that provides energy for the cell to use through the synthesis of adenosine triphosphate (ATP). Mitochondrial ATP synthase could affect gametophyte development (Hanson and Bentolila 2004) and its subunit ORFB has been shown previously to be associated with cytoplasmic male sterility (CMS) in plants (Sarbar *et al*. 2003). Chlorophyll a/b binding polypeptides are essential for photosynthetic light harvesting (Hoffman *et al*. 1987), and the chlorophyll a/b binding gene expression was involved in response to hormones (Chang and Walling 1991, Teyssendier de la Serve *et al.* 1985). Expression of Chlorophyll a/b binding protein genes were negatively regulated by abscisic acid during soybean embryogeny (Chang and Walling 1991) and were greatly affected by cytokinin in tobacco cells (eyssendier de la Serve *et al.* 1985). Succinyl-CoA synthetase was the key enzyme in tricarboxylic acid cycle; it catalysis Succinyl-CoA to sucinnate, generating high-energy phosphate bonds at the same time. Succinyl-CoA synthetase activity was increased in the etiolated bean leaves when exposed to light (Steer and Gibbs 1969).

Energy metabolism is fundamental to biological processes. In our study, protein related to photosynthesis chloroplast chlorophyll a/b binding protein (spot 6) and the Succinyl-CoA synthetase (spot 27) were up regulated, the two proteins were related to photosynthesis and light condition, consistent with the growth trend and metabolism regulation treated by CPPU. Energy metabolism is also a dynamic balancing process, ATP synthase (spot 8) down-regulated by CPPU may be play a role in a balanced energy in the male *V. amurensis* sex conversion.

Proteins related to pollen tube growth were inhibited in sex conversion: Pollen tube growth was regulated by many proteins in different processes, such as ubiquitin/proteasome pathway, cytoskeletal elements, membrane fusion and vesicular trafficking. The 20S proteasome is considered as an essential component of ubiquitin/proteasome pathway in eukarya which substantially influences much of plant biology, including cell division, hormone responses, biotic and abiotic stress responses and plant development (Smalle and Verstral 2004). Inhibitors of proteasome activity could significantly prevent pollen tube development and markedly alter tube morphology, and the pathway seems to be involved in the degeneration of female kiwifruit pollen (Scoccianti *et al*. 1999, Speranza *et al*. 2001), which implicates that it is a major regulator of sexual reproduction and contributed significantly to development by affecting hormone signaling. Microtubules and actin filaments are both present in virtually all eukaryotic cells as major components of cytoskeleton (Fosket 1992, Pollard and Cooper 2009), and contributes to biological processes such as cell morphogenesis, response to environmental factors, and cell division (Pollard and Cooper 2009, Wasteneys 2004). The dynamic involvement of cytoskeletal elements (actin, microtubules) in pollen germination and tube growth was discussed and the results indicated both of these were essential for this process (Taylor and Hepler 1997). Soluble NSF-attachment proteins (SNAPs) are essential for intracellular membrane fusion and vesicular trafficking (Stenbeck 1998). A recent study implicates a possible role of SNAPs in pollen tube growth because a member of the soluble NSF attachment protein receptor (SNARE) family which is closely connected to SNAPs, mediates fusion of endo- and exocytic compartments in pollen tube tip growth (Guo and McCubbin 2012). This hypothesis is supported by previous research on the effect of α-SNAP on priming and triggering of regulated exocytosis (Chamberlain *et al*. 1995). So we presume that alpha-soluble NSF attachment protein (α-SNAP) possibly regulates pollen tube growth by balancing exo- and endocytic processes.

In the present study, all of the proteins related to pollen tube growth were down-regulated by CPPU treatment, so 20S proteasomes (spot 1 and 16) perhaps receive the CPPU's signal and transfer to cytoskeletal elements proteins (spot 14 and 17) and soluble NSF-attachment protein (spot 15). Male grape flower repressed pollen growth through the inhibition of proteasome function, cytoskeletal development and balance exo- and endocytic processes under the CPPU treatment.

Proteins related to cell elongation was promoted in sex conversion: The ribosome-associated protein is on the 40S ribosome in the nucleus. Wu *et al*. (2006) have analyzed the function by knocking-out or knocking-down their expression in rice, which resulted in retarded cell elongation with smaller size or shorter length in all vegetative and reproductive organs. Additionally, kiwifruit can be enlarged by application of CPPU through stimulating cell division and/or cell expansion (Kim *et al*. 2006). To support this results, ribosome associated protein p40 (spot 23) was up-regulated by CPPU and identified successfully in this study, this up-regulation may be related to the promotion of cell elongation, evidenced by longer stems, and larger fruits.

Protein related to flower sex was up-regulated in the sex conversion process: Adenine is converted to adenosine monophosphate (AMP) by adenine phosphoribosyl transferase (APT) in the purine salvage pathway (Xing *et al*. 2005), and ATP has a possible role in cytokinin metabolism (Moffatt *et al*. 1991). A mutant of *Arabidopsis thaliana* deficient in APT activity was shown to be responsible for male sterility, and APT may affect pollen development by altering the level of cytokinin in some ways in plant cells (Moffat and Somerville 1988). Significantly, in *Vitis*, the gene encoding an APT with the highest homology to the *APT3* gene from *Arabidopsis thaliana* was recently revealed by transcriptome analysis as one of the candidate genes with a potential impact on flower sex formation in *Vitis* (Fechter *et al*. 2012). Adenine phosphoribosyl transferase 3 (spot 10) was upregulated in the later stages of male *V. amurensis* sex conversion in our

study. Therefore, we presumed that CPPU application possibly upregulated the expression of flower sex gene and it might convert flower sex through regulating cytokinin level. Furthermore, hormones have the ability of reversing the sex determination mechanism in several dioecious plants, such as in *Spinacia*, and *Cannabis* (Chailakhyan and Khryanin 1978, Galoch 1978).

## Conclusions

In conclusion, this is the first report on the comparative proteomic analysis of sex conversion in wild grape. Most of the proteins involved in male *V. amurensis* sex conversion directly or indirectly participate in flower growth and development. It is possible that CPPU may regulate the flower sex conversion by ROS signal in the beginning. With the energy balance regulation in the whole process, stamens may be repressed though the inhibition of pollen tube development proteins, while the pistils were promoted by the up-regulated cell elongation proteins. Adenine phosphoribosyl transferase 3 may also be the key enzyme in the sex conversion and continuously increasing in the later stages, so adenine phosphoribosyl transferase 3 possibly is the sex- specific protein of *V. amurensis* and could act as molecular marker in molecular breeding of grapevine. For better understanding of the above results, molecular biological analysis and other subcellular proteomic studies should be performed in the future.

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