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The Effect of Hydrogen Cyanamide on Dormancy Breaking In Grapevine Buds: Reactive Oxygen Species Accumulation and Related Genes Expression

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Abstract - Hydrogen cyanamide (HC) and pruning have frequently been used to break dormancy in grapevine floral buds. This study aimed to address the effects of these treatments on accumulation of ROS and alteration in expression of ROS-related genes in the dormancy breaking buds of grapevine in the summer in sub/tropical. Four treatments were compared, namely: Pruning (P), hydrogen cyanamide (HC), Pruning and hydrogen cyanamide (PHC) and Control (water) and 8 days after treatment the bud break rates were 33%, 53%, 95%, and 0%, respectively. Clearly, HC is effective in stimulating grapevine bud break and pruning further enhanced its potency. In situ detection of various Reactive oxygen species (ROS) and Nitric oxide (NO) in longitudinal bud sections after 12 h of treatments showed that high levels of ROS and NO accumulate in the buds treated with PHC, compared with HC or P only. The amounts of ROS and NO accumulated were closely correlated with the rates of bud break among the treatments. Microarray analysis was conducted with the dormancy breaking buds after 24 h of treatments. Gene ontology (GO) analysis indicated that alteration in expression of ROS related genes is the major factor responsible for bud break. PHC treatment gave rise to dynamic changes in highly up-/down-regulation of antioxidant activity at 24 h post-treatment. Twelve genes were identified as key genes involved in dormancy bud break in the early response. The time course of expression of these genes (examined by qRT-PCR) showed different expression during the 48 h treatment. It is concluded that accumulation of ROS at the early stage is important for dormant bud break, and that the microarray analysis of differentially expressed genes among the treatments allowed the construction of the model pathway related to ROS metabolisms during dormant bud breaking.

Keywords**:** Hydrogen cyanamide, ROS, dormancy breaking buds, grapevines, Gene Ontology

1. Introduction

Grapevine is a perennial crop in temperate climates and perceives cool temperatures as a signal to onset bud endodormancy while entering winter; subsequently it requires chilling temperature for several weeks (or months) to end of endodormancy of these buds [1]. Grapevine grown in subtropical regions, often have an uneven floral bud break in early spring due to warm winter that providing inadequate chilling. Fortunately, HC have been found to be one of the most useful compounds for breaking dormancy in floral buds in grapevine [2-4] , kiwifruit [5] and apple [6, 7]. In subtropical regions the use of HC treatment on intact dormant buds in spring is an important grapevine orchard management tool to regulate for

even floral bud break. This treatment is also very useful in Taiwan for the second grapevine harvest in December, where paradormant buds on the prune canes can be treated with HC to ensure effective bud break even in hot August.

To gain a better understanding of the underlying mechanism of bud break by pruning and/or HC treatment, this study aimed to observe the burst out of endogenous ROS and NO levels in the dormancy breaking buds by cytochemical staining and to identify the sets of candidate genes being significantly altered by microarray analysis. Go categories that related to ROS-generating, ROS- scavenging, such as peroxidase activity (GO:0004601), antioxidant activity (GO: 0016209) and NO detoxification were identified as key genes involved in breaking dormancy of grapevine buds in early response and their expression patterns during treatment were subsequently analysed by qRT-PCR analysis.

2. Materials and Methods

Plant material

The grapevine buds used in this experiments in August were collected from the mature canes of 5-year-old plants (V. vinifera. x V. labruscana Bailey cv. Kyoho) grown in the research vineyard of the Taichung District Agricultural Research and Extension Station (Taichung DARES, 24º00'N, 120º53'E, elevation19 M), Taiwan. Uniform grapevine canes were used for four treatments with water only (as control; CK), pruning only (P), 1% (w/v) of HC (Dormex, SKW, Trostberg, Germany) and a combination of pruning and HC (PHC).

Observation of bud developments after treatments by light microscopy

Grapevine buds were collected before and after treatments and fixed in FAA solution for 2 h, stained with filtered 0.1% Harris Hematoxylin solution, Images of sections were captured.

In situ H2O2, O2$^{\bullet-}$ and NO detection by fluorescence microscopy

For *in situ* staining assay of H_2O_2, $O_2^{\bullet-}$ and NO, dormant grapevine buds treated with P, HC, PHC and CK for 12 h were sectioned (10μm thickness) and stained for H_2O_2, $O_2^{\bullet-}$ and NO For H_2O_2 assay, sections were stained with 25μM 2´, 7´-dichlorofluorescin diacetate (DCF-DA, Calbiochem, San Diego, CA, USA) [8]. For $O_2^{\bullet-}$ assay, sections were stained with 10μM dihydroethidium (DHE, Calbiochem, San Diego, CA, USA) [9]. For NO on the sections were stained with 10μM 4, 5-diaminonaphthalene (DAF-2 DA, Calbiochem, San Diego, CA, USA) prepared in 10 mM Tris- HCl (pH 7.4). For negative controls, sections were incubated sequentially with 1 mM sodium pyruvate (H_2O_2 scavenger), 1 mM tetramethylpiperdinooxy ($O_2^{\bullet-}$ scavenger), and 1 mM carboxy-PTIO(NO inhibitor).

RNA extraction and microarray analysis

Microarray experiment procedures were carried out following the manufacturer's protocols. Briefly, 1 μg of total RNA was amplified by a Agilent Qiuck Amp Labeling Kit (Agilent Technologies,USA) and labeled with Cy3-CTP or Cy5-CTP (Agilent Technologies, USA). Correspondingly fragmented labeled cRNA is then pooled and hybridized to Agilent Whole Grape Genome 4x44k oligo microarray (Agilent Technologies, USA). Scanned images are analyzed by Feature extraction software 10.5(Agilent Technologies, USA), finally, the GenSpring software (Agilent Technologies, USA) is used to identify of significant differentially expressed genes (DEGs).

Differentially expressed gene annotation and functional categorization

Gene Ontology (GO) analysis for gene information annotation and functional category distribution frequency was performed using the ErmineJ (version 3.0.2) software [10].

Quantification of gene expression by quantitative RT- PCR (qRT-PCR)

Total RNA was extracted at 0, 6, 12, 24, 48 h post-treatment and 12 grapevine genes were selected for quantification of gene expression by quantitative RT-PCR.

3. Results and Discussion

Dormant bud break rates

In this study we showed that intact pruned (P) in combination with HC (PHC) treatment effectively alleviate the dormancy of grapevine buds and result in bud break and floral development in hot summer (Figure 1A). PHC induces bud break more rapidly within 8 days and efficiently (95%) than P or HC treatment alone, indicating a synergetic interaction between P and HC treatments (Figure 1B). A similar result has been reported previously in grapevine [11]. Compared to dormant buds, PHC treated buds showed a bud break mediated by paradormancy, in which growth is regulated by plant growth regulators originating outside the bud affecting apical dominance [12]. In contrast to paradormancy that occurs in the summer, endodormancy that occurs during winter is a major problem for grape production in warm winter region [13, 14].

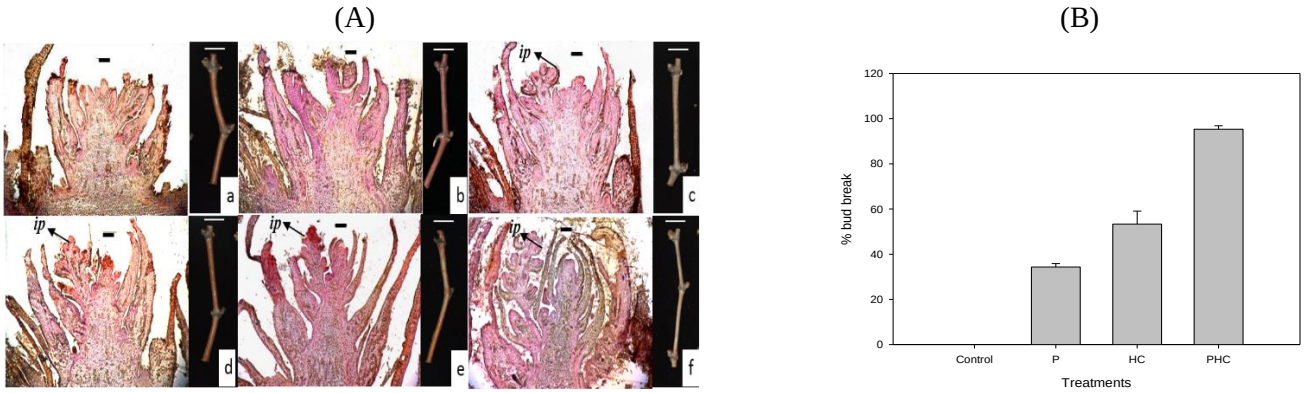

Fig. 1: Morphological observation and anatomical examination of grapevine bud break. (A) Longitudinal sections of grapevine floral development bud during breaking of dormancy after PHC treatment. (a) control (0 h), (b) 6 h, (c) 12 h, (d) 24 h, (e) 48 h and (f) 96 h. ip: inflorescence primordium. Bar: 200 µm. (B) Percentages of bud break after 192 h of treatment with P, HC, PHC, or water as a control (n = 10, Bar: standard deviation).

In situ H_2O_2, $O_2^{\bullet-}$ and NO detection by fluorescence microscopy

In this study, we detected large amounts of H_2O_2, $O_2^{\bullet-}$ and NO levels accumulated rapidly in bud tissues by *in situ* localization upon the treatments, especially in PHC treated buds (Figure 2).

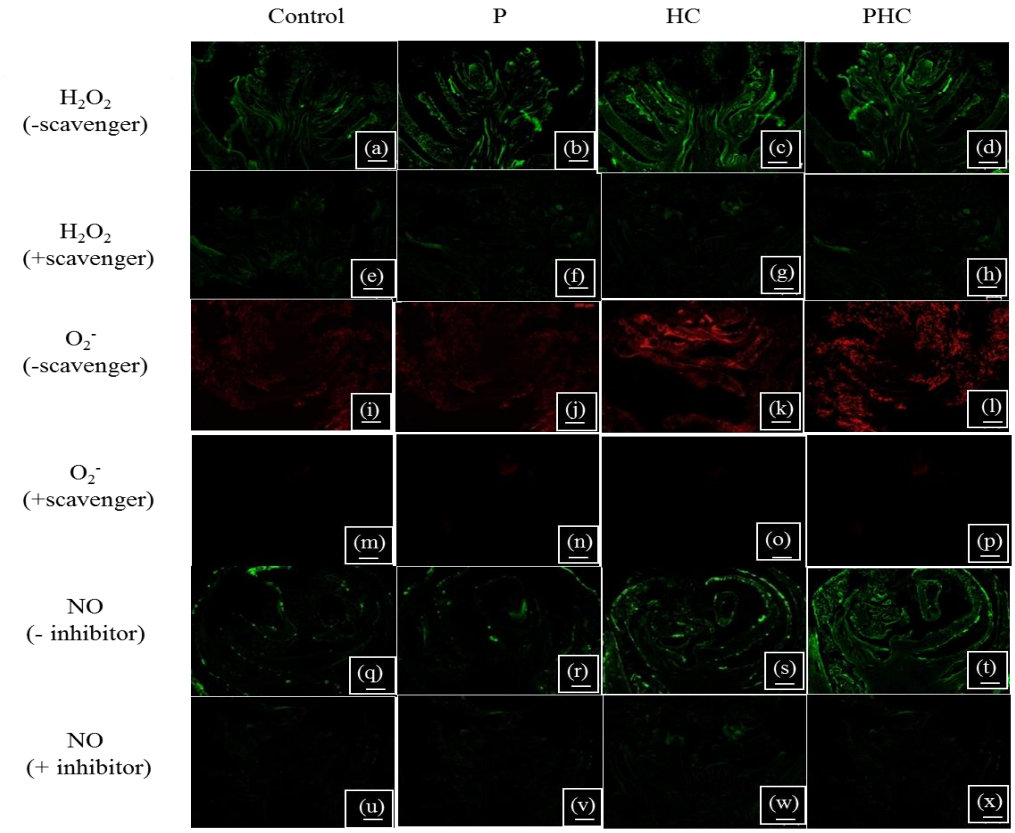

Fig. 2: Detection of H2O2, O2•- and NO at early stage of grapevine bud break.

Visualization of H2O2 by fluorescence microscopy using DCF-DA assay in grapevine bud sections after 12 h of treatment with H2O or control (a), P (b), HC (c) and PHC (d). For negative control, grapevine bud sections were incubated with 1 mM sodium pyruvate, an H2O2 scavenger: control (e), P (f), HC (g) and PHC (h). Visualization of O2•- by reaction

with 10μM dihydroethidium (DHE) in grapevine bud sections: H2O or control (i), P (j), HC (k) and PHC (l). For negative control, grapevine bud sections were incubated in 1 mM tetramethylpiperdinooxy, an O2•- scavenger: control (m), P (n), HC (o) and PHC (p). Visualization of nitric oxide (NO) by DAF-2DA assay in grapevine bud sections: control (q), P (r), HC (s) and PHC (t). For negative control, grapevine bud sections were incubated in 10 μM carboxy-PTIO, an NO inhibitor: control (u), P (v), HC (w) and PHC (x). Bar: 200 μm.

Classification of the expression of up/down regulated genes that related to ROS in three treatment at 24 post-treatment

The expressed genes that were significantly regulated by the three treatments were identified by GenSpring. It is noteworthy that a total of 1148, 2251 and 4058 genes showed up-regulated expression and a total of 206, 1169 and 3115 genes showed down-regulated expression by P, HC and PHC treatments, respectively. Clearly, alteration in expression of many genes is involved in dormant bud break in grapevine and many more genes are up-regulated than down-regulated by these treatments.

GO classification of ROS related genes that up-/down regulated in molecular function at 24 h post-treatment, these genes showing altered expression in ROS- and NO-related. We identified clear overlaps of the molecular functions among the up-/down-regulated genes in responses to P, HC and PHC. GO term were showed its individual expression levels by significantly at least two fold in up-/down-regulated changes in each treatment.

However, PHC treatment was mostly induced up-/down-regulated genes following HC and P treatment. The specific of P and HC response are suggests that there are common regulation of genes response to PHC. ROS-generating genes; respiratory burst oxidase homolog protein E; VvRBOHE, respiratory burst oxidase homolog protein A; VvRBOHA, VvPOD72, VvPOD12 and VvDOX were identified. Two-VvRBOH were appeared in binding (GO:0005488), which both VvRBOHE and VvRBOHA were showed down-regulated of their expression in PHC, and one-VvRBOH was down-regulated in HC. VvPOD and VvDOX were appeared in peroxidase activity (GO:0004601), which the VvPOD genes; that mention are ROS-generating genes have been expressed in up-/down-regulated genes in P (10/1), HC (10/14) and PHC (15/10). One VvDOX gene was up-regulated in P and HC, which was not found in PHC. For ROS-scavenging genes were including in antioxidant activity (GO:0016209), which totally four genes family have been expressed in up-/down regulated along three treatments; such as one glutathione peroxidase; VvGPX gene was up-regulated in PHC and the another one VvGPX gene was down-regulated in HC and PHC, while two catalase isozyme –like; VvCAT genes were down-regulated in both HC and PHC. In addition, cytosolic ascorbate peroxidase; VvAPX was found in down-regulated in HC and PHC, superoxide dismutase [Fe], chloroplastic; VvFSD3 was down-regulated in HC and PHC and VvFSD was only up-regulated in PHC. VvAOX was up-regulated in HC and PHC. For NO relative gene, hemoglobin-2; VvHB2 was including in binding (GO:0005488) by up-regulated expression in HC and PHC. ROS-transcription factor, four gene families; heat stress transcription factor; VvHSF, ethylene responsive transcription factor; VvERF and WRKY transcription factor; VvWRKY were found in transcription factor (GO:0003700). The number of VvHSF genes were up-/down-regulated by P (0/1), HC (2/2), and PHC (3/6), while the VvERF genes were up-/down-regulated expressed in P (4/3), HC (8/6), and PHC (8/9). VvWRKY genes were up-/down-regulated in P (1/0), HC (9/4), PHC (7/7), respectively.

Detection of ROS-generating, ROS- scavenging and NO-scavenging genes

Consistent with this observation, the expression of genes coding for five key ROS-generating genes, were both induced (VvPOD72, VvDOX) and suppressed (VvRBOHE, VvRBOHA, VvPOD12) at the early stage of PHC treatment. In addition, ROS-scavenging genes, such as VvCAT and VvFSD3 were suppressed at the early stage of PHC treatment (Fig 3), thus allowing the rapid accumulation of related ROS.

As reported in grape buds after 12-24 h of hydrogen cyanide treatment was reduced *CAT* activity and expression, but was induced up-regulated of oxidative stress related genes, such as *Trxh, GST, APX, GR*, and hypoxia related genes, such as *SuSy* [4, 15, 16]. *A* common mode of ROS regulation gene networks and its modulation in grapevine dormant buds break are identified their expression profiles during PHC treatment by qRT-PCR. Totally 12 genes were identified by qRT-PCR; six genes were related to ROS-scavenging genes, such as *VvFSD3* and *VvAOX* are turned on and expressed at high level at 6 h, while *VvGPX* and *VvAPX* were turned on and expressed at high level as early as 12 h of treatment and decline at 24-48 h, except *VvCAT* and *VvFSD3* which were down-regulated.

For RNS detoxification we have found the expression of *VvHB2* is highest expression at 6 h post-treatment by PHC, which *AHb1* is a member of the class I family of nonsymbiotic hemoglobins [17], and its possessing activity against NO [18] and AHb1 expression is induced in roots and rosette leaves by low O2 levels [19]. Therefore, we recommend the *VvHB2*

was early highest expression at 6 h post-treatment which can be inhibited the NO accumulation that further be able damaging the cell.

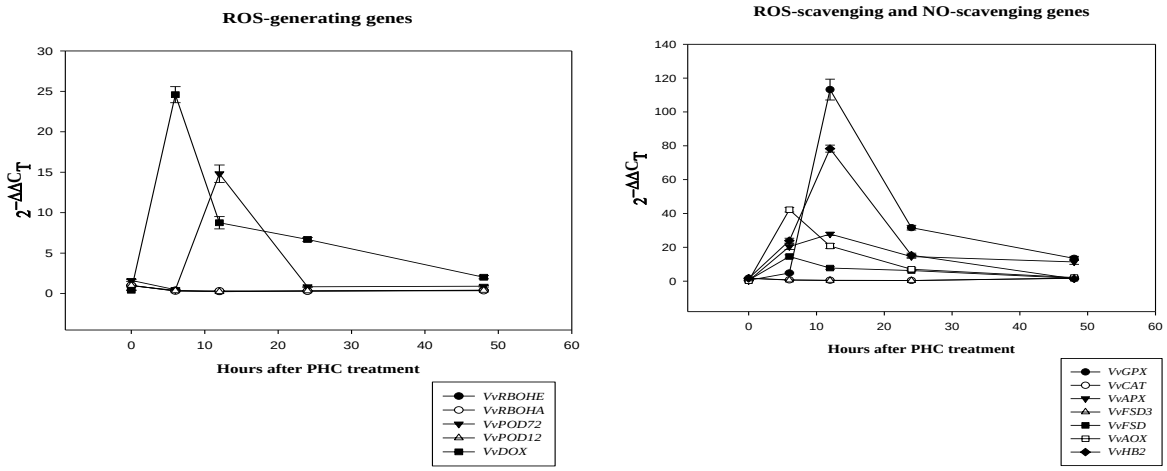

Fig. 3: The gene expression among the 12 up-/down-regulated ROS-relative genes induced by PHC. During of 48 h treatment, as analyzed by qRT-PCR, ROS-generating genes; (A) VvRBOHE, VvRBOHA, VvPOD72, VvPOD12 and (B) VvDOX. ROS-scavenging genes; VvGPX, VvCAT, VvAPX, VvFSD3, VvFSD, VvAOX and NO-scavenging genes; VvHB2.

4. Conclusions

In concluded, this study is contributed our understanding to underlying in ROS metabolism pathway. According to hydrogen cyanamide is applied to grapevine dormant buds, its need ROS-mediated to breaking dormant buds. The upstream events, likely ROS generating and ROS accumulation in the cell were induced the oxidative stress and trigger programmed cell death response in the early stage (e.g. 6-12 h) of hydrogen cyanamide treatment in grapevine buds. In addition, the interaction of ROS and NO can be correlated in activating dormancy breaking in grapevine buds.

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