J Biosci (2019) 44:136
DOI: 10.1007/s12038-019-9949-4

Involvement of putrescine in osmotic stress-induced ABA signaling in leaves of wheat seedlings

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MS received 20 March 2019; accepted 16 July 2019; published online 24 October 2019

To elucidate one mechanism by which putrescine (Put) functions in plant signaling under osmotic stress, Put and ABA contents, and plasma membrane-NADPH oxidase (PM-NOX) activity were detected in wheat seedling leaves. Under osmotic stress, ABA and Put contents, PM-NOX activity, and PM-NOX-dependent $O_2^{\cdot-}$ production all increased. The inhibitor tungstate (T) of ABA bio-synthesis reduced the increases in ABA and Put contents under osmotic stress. The inhibitor D-arginine (D-Arg) of Put bio-synthesis didn't reduce osmotic-induced increase of ABA, but it inhibited the increases of PM-NOX activity and $O_2^{\cdot-}$ production, and the inhibitory effects were reversed by exogenous Put. These findings suggested that ABA might regulate Put biosynthesis, and Put might regulate PM-NOX activity. Treatments with three inhibitors imidazole (I), diphenylene iodonium (DPI) and pyridine (P) of PM-NOX reduced significantly not only $O_2^{\cdot-}$ production, but also the stress-induced increase of Put content, which indicated that $O_2^{\cdot-}$ production might regulate Put biosynthesis. Treatments with EGTA (Ca^{2+} chelator), La^{3+} and verapamil (V) (Ca^{2+} channel blockers) reduced significantly the stress-induced increase of Put content, which suggested that Ca^{2+} might regulate Put biosynthesis. With these findings, it could be concluded that Put was involved in ABA signaling induced by osmotic stress via regulating PM-NOX activity in wheat seedling leaves.

Keywords. Osmotic stress; putrescine; signaling; wheat (*Triticum aestivum* L.)

1. Introduction

In plants, water deficit inhibits significantly cell division and expansion (Avramova *et al*. 2015). Under water deficit circumstance, cell membrane receptors sense the deficit signal and transmit it to the cell inner, then triggering a series of bio-chemical reactions (Zhu 2016). Plasma membrane (PM)-NADPH oxidase (NOX) is involved in abscisic acid (ABA) signaling pathway by osmotic stress in maize seedlings (Jiang and Zhang 2002, 2003) and heavy metal stress (Hao *et al*. 2006). In the past researches, it has been extensively documented that ABA induced antioxidant defense (Wang *et al*. 2006; Zhu 2002, 2016). For example, ABA could accelerate the increase of reactive oxygen originated from PM-NOX (Guan *et al*. 2000; Pei *et al*. 2000), and ABA-dependent signaling pathways are involved in the expression of up-regulation genes of antioxidant enzymes under osmotic stress (Guan *et al*. 2000). Calcium (Ca^{2+}) has also been involved in ABA signal transduction pathway in plant cells (Pei *et al*. 2000; Murata *et al*. 2001). ABA promoted cytosolic Ca^{2+} increase induced by both Ca^{2+} influx from the extracellular and Ca^{2+} release from intracellular (Pei *et al*. 2000). The study of Jiang and Zhang (2003) suggested that a cross-talk between Ca^{2+} and reactive oxygen originated form PM-NOX was involved in the ABA signal transduction pathway (ABA→PM-NOX→$O_2^{\cdot-}$→H_2O_2→Ca^{2+}→…).

Polyamines (PAs) are aliphatic nitrogenous amines and are ubiquitously existed in plants. They are closely related to growth and development of plants (Du *et al*. 2017; Guo *et al*. 2018). In plants, PAs mainly include putrescine (Put), spermidine (Spd), spermine (Spm). D-arginine (D-Arg) is an inhibitor of Put biosynthesis. A lot of studies indicated that PAs in plants are closely related with osmotic stress (Liu *et al*. 2005a; Du *et al*. 2015; Pál *et al*. 2018). However, the mechanism by which PAs enhance the tolerance of plant to stress is not yet clear. Researchers revealed that PAs in cell wall were oxidized by polyamine oxidase (PAO) to form H_2O_2, which triggers the hypersensitive response (Cona *et al*. 2003). Liu *et al*. (2000) reported that PAs targeted KATI-like inward K^+ channels in

guard cells and modulated stomatal movement. Recently, PAs being mediated phosphorylation (Gupta *et al*. 2012) and interaction between PAs and nitric oxide signaling (Montilla-Bascón *et al*. 2017) have been documented. However, relationship between PA and osmotic stress-induced ABA signaling remains to be investigated.

The study aimed to elucidate the significant of Put in wheat seedling leaves in osmotic stress-induced ABA signaling, including the following items: relationship between PA and ABA, between PA and PM-NOX-dependent $O_2^{\cdot -}$ production, and between PA and Ca^{2+}.

2. Materials and methods

2.1 *Wheat seedling cultivation and treatments*

Wheat (*Triticum aestivum* L. Zhoumai No. 27 cultivar, from Zhoukou Academy of Agricultural Sciences) seeds were selected and sterilized in 0.2% (w/v) $HgCl_2$ solution for 10 min, then washed with distill water for three times, and germinated in culture dishes (diameter: 15 cm) with germination bed designed in three layers of filter paper. After these seeds had germinated and grown up for 7 d, the seedlings were planted in plastic teacups with little pores on the bottoms containing 3/4 sands of a teacup volume, and then the plastic teacups were put in plastic trays (length × width × high: 30 × 20 × 10cm) and cultivated with half-strength Hoagland's solution in a controlled greenhouse at a temperature of 22/15°C (day/night), with a photosynthetic active radiation (PAR) of 400 μmol m^{-2} s^{-1} and a photoperiod of 14/10 h (day/night). The solution was renewed per 2 days. When the third leaf of the wheat seedlings was fully expanded, the seedling roots were treated for 12 h with Hoagland's solution containing the following reagents, respectively (1) PEG (15%, −0.33 MPa); (2)PEG (15%)+D-Arg (0.5 mM); (3) PEG (15%)+D-Arg (0.5 mM)+Put (0.5 mM); (4)PEG (15%)+ tungstate (T, 1 mM); (5)PEG (15%)+DPI (20 μM) or imidazole (I, 10 mM) or pyridine (P, 10 mM);(6)PEG (15%)+EGTA (2 mM) or $LaCl_3$ (2 mM) or verapamil (V, 0.5 mM).

The control seedlings remained well watered (normal moisture conditions) without the reagents mentioned above. After 12 h treatment, the leaves of the treated and control seedlings were clipped and tested for the subsequent experiments. Put, D-Arg, tungstate, imidazole, pyridine, DPI, EGTA, $LaCl_3$, and verapamil were purchased from Sigma Chemical Co. (USA).

2.2 *Plasma membrane separation and protein determination*

Leaf samples were ground and homogenized using extraction buffers. The homogenate was filtered using 0.45 um filtering membrane and the filtrate was centrifuged at 12000*g* for 35 min. The plasma membranes of the wheat seedling leaves were separated using the two-phase aqueous polymer partition system according to Du *et al.* (2015). The protein level from PM was quantified using the method described by Bradford (1976) with bull serum albumin (BSA) as standard.

2.3 *Measure of PM-NOX activity*

PM separated above was used to detect the PM-NOX activity according to Sagi and Fluhr (2001) and Jiang and Zhang (2003). The NOX activity was evaluated by the reduction of XTT.

2.4 *Measure of PM-NOX-dependent $O_2^{\cdot -}$ production rate*

The production rate of PM-NOX-dependent $O_2^{\cdot -}$ was evaluated according to the method described by Sagi and Fluhr (2001) and Jiang and Zhang (2003).

2.5 *Measure of Put content*

Put in wheat seedling was extracted according to Liu *et al.* (2005a) and quantified by HPLC (Waters 2696, US) at 254 nm.

2.6 *Determination of ABA content*

The wheat leaves were washed with distilled water and weighted for 1 g. Then, the leaves (1 g) were ground and homogenized in 80% methanol. The homogenate was centrifuged for 30 min at 11000*g*. The supernatants were eluted by the Sep-Pak C18 cartridge (Waters, USA) to removal of polar compounds, and quantified by enzyme linked immune sorbent assay as described by Zhang *et al.* (2017).

2.7 *Data statistic analysis*

The experiments were repeated three times and three samples were taken for every experiment. Data were analyzed using SPSS16.0 and Microsoft Excel software. Every value in figures or tables reported in this paper is mean (n =9) ± stand error (SE). The significant differences among different treatments were determined using by Duncan's multiple range tests at a significance level ($P<0.05$).

3. Results

3.1 *Effect of PEG, D-Arg and exogenous Put on Put contents in wheat seedling leaves*

PEG-6000 treatment for 12 h led to the increase in Put content from 97 to 173 nmol g^{-1} FW in wheat seedling

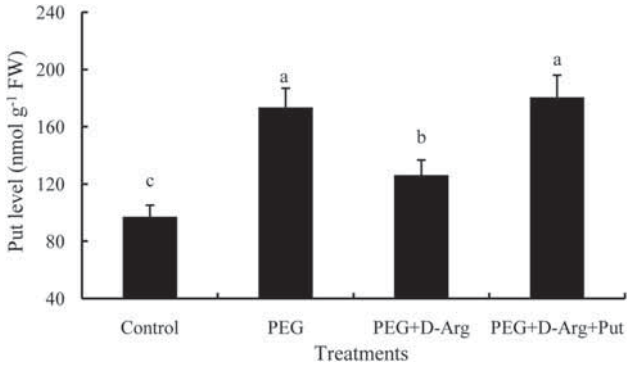

Figure 1. Effects of PEG, D-Arg and exogenous Put on Put content in leaves of wheat seedlings. Control, seedlings remained well-watered (normal moisture conditions) without PEG, D-Arg, or Put; PEG, osmotic stress treatment; D-Arg, D-Arginine (an inhibitor of Put biosynthesis). Each value in the figure represents the mean of three experiments ± SE. Error bars indicate SE (n = 9), and different letters (a-c) above the columns are significantly different by Duncan's multiple range tests ($P < 0.05$).

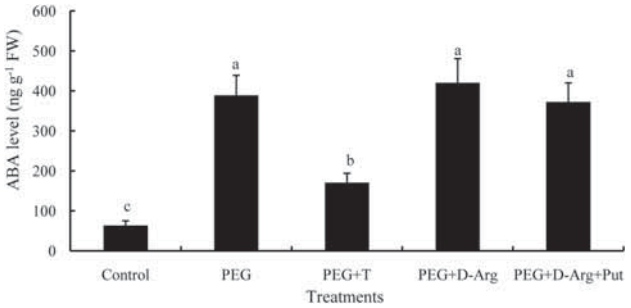

Figure 2. Effects of PEG, D-Arg, exogenous Put and T on ABA content in leaves of wheat seedlings. Control, seedlings remained well-watered (normal moisture conditions) without PEG, T, D-Arg, or Put; PEG, osmotic stress treatment; T–tungstate (an inhibitor of ABA biosynthesis); D-Arg, D-Arginine (an inhibitor of Put biosynthesis). Each value in the figure represents the mean of three experiments ± SE. Error bars indicate SE (n = 9), and different letters (a-c) above the columns are significantly different as determined by Duncan's multiple range tests ($P < 0.05$).

leaves. D-Arg, an inhibitor of Put biosynthesis, reduced the PEG stress-induced increase of Put from 173 to 126 nmol g^{-1} FW, and exogenous Put treatment reversed the effect of D-Arg on Put level (figure 1). These results verified that D-Arg inhibited the Put biosynthesis.

3.2 *Effect of PEG, D-Arg, exogenous Put and T on the ABA content in wheat seedling leaves*

PEG treatment brought about the significant increase of ABA content from 63 to 389 ng g^{-1} FW, and treatment with T, an inhibitor of ABA biosynthesis, markedly reduced the ABA content from 389 to 170 ng g^{-1} FW. In order to elucidate whether Put modulates ABA biosynthesis, exogenous Put and D-Arg (an inhibitor of Put biosynthesis) were used. The results showed that Put or D-Arg scarcely affected the ABA content (from 389 to 372 and to 420 ng g^{-1} FW) in wheat seedling leaves subjected to osmotic stress (figure 2), which suggested that ABA biosynthesis was not modulated by Put.

3.3 *Effect of PEG, D-Arg and exogenous Put on the PM-NOX activity and PM-NOX-dependent O_2^- production in wheat seedling leaves*

Osmotic stress treatment promoted the increases of PM-NOX activity and PM-NOX-dependent O_2^- production from 1.7 to 5.7 nmol mg^{-1} protein min^{-1} and 0.33 to 1.21 nmol mg^{-1} protein min^{-1} in wheat seedling leaves, respectively (figure 3). D-Arg treatment reduced the stress-induced increases from 5.7 to 3.9 nmol mg^{-1} protein min^{-1} and 1.21 to 0.78 nmol mg^{-1} protein min^{-1}, respectively, and exogenous Put reversed the inhibitory effects caused by D-Arg (figure 3), which indicated that the increases induced by osmotic stress in the PM-NOX activity and PM-NOX-dependent O_2^- production were partly attributed to the increase in Put content.

3.4 *Effect of DPI, I and P on the PM-NOX activity and PM-NOX-dependent O_2^- production in wheat seedling leaves under osmotic stress*

Under osmotic stress, the PM-NOX activity and PM-NOX-dependent O_2^- production increased by 335% and 366%,

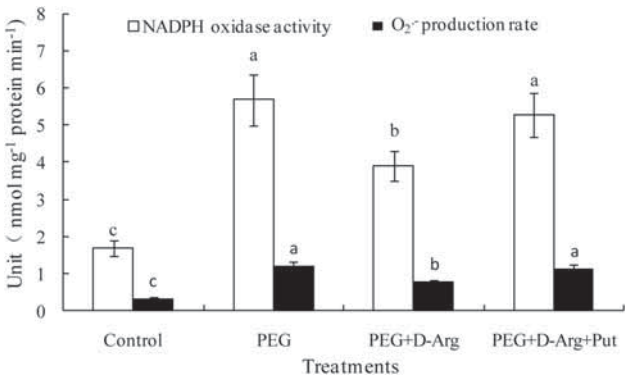

Figure 3. Effects of PEG, D-Arg and exogenous Put on PM-NOX activity and PM-NOX-dependent O_2^- production rate in leaves of wheat seedlings. Control, seedlings remained well-watered (normal moisture conditions) without PEG D-Arg, or Put; PEG, osmotic stress treatment; D-Arg, D-Arginine (an inhibitor of Put biosynthesis). Each value in the figure represents the mean of three experiments ± SE. Error bars indicate SE (n = 9), and the different letters (a-c) above the columns are significantly different as determined by Duncan's multiple range tests ($P < 0.05$).

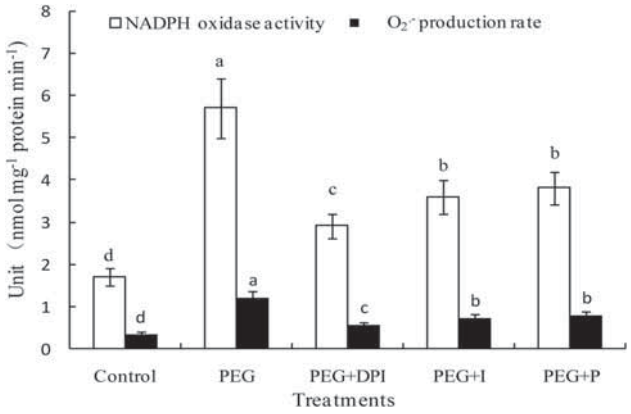

Figure 4. Effects of PEG, DPI, I and P on activity of PM-NOX and PM-NOX-dependent $O_2^{\cdot-}$ production rate in leaves of wheat seedlings under osmotic stress. Control, seedlings remained well-watered (normal moisture conditions) without PEG, DPI, I, or P; PEG, osmotic stress treatment; DPI–diphenylene iodonium (an inhibitor of PM-NOX); I, imidazole (an inhibitor of PM-NOX); P, pyridine (an inhibitor of PM-NOX). Each value in the figure represents the mean of three experiments ± SE. Error bars indicate SE (n = 9), and different letters (a-d) above the columns are significantly different as determined by Duncan's multiple range tests ($P < 0.05$).

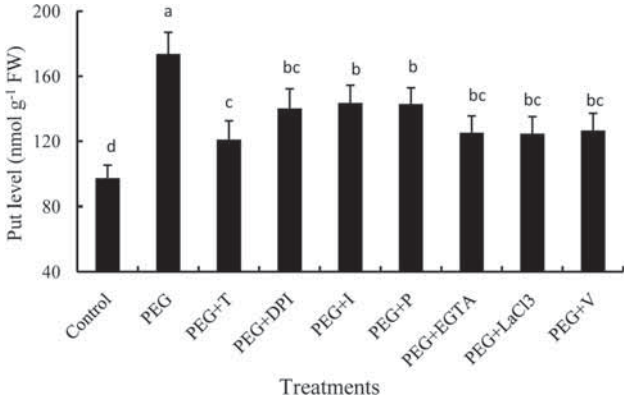

Figure 5. Effects of T, DPI, I, P, EGTA, $LaCl_3$ and V on Put content in leaves of wheat seedlings under osmotic stress. Control, seedlings remained well-watered (normal moisture conditions) without PEG, DPI, I, P, EGTA, $LaCl_3$, or V; PEG, osmotic stress treatment; T, tungstate (an inhibitor of ABA biosynthesis); DPI, diphenylene iodonium (an inhibitor of PM-NOX); I, imidazole (an inhibitor of PM-NOX); P, pyridine (an inhibitor of PM-NOX); EGTA, ethylene glycol-bis (2-aminoethyl ether)- N,N,N',N'-tetraacetic acid (Ca^{2+} chelator); $LaCl_3$, $LaCl_3$ (Ca^{2+} channel blockers); V, verapamil (Ca^{2+} channel blockers). Each value in the figure represents the mean of three experiments ± SE. Error bars indicate SE (n = 9), and different letters (a-d) above the columns are significantly different as determined by Duncan's multiple range tests ($P < 0.05$).

respectively (figure 4). However, the treatments with three inhibitors (DPI, I and P) of NOX significantly inhibited the increases in the PM-NOX activity from 5.5 to 3.7 nmol mg^{-1} protein min^{-1} or so and PM-NOX-dependent $O_2^{\cdot-}$ production from 1.2 to 0.7 nmol mg^{-1} protein min^{-1} or so (f 4), which verified that DPI, I and P actually inhibited the PM-NOX activity.

3.5 *Effect of T, DPI, I, P, EGTA, $LaCl_3$ and V on the Put content in wheat seedling leaves under osmotic stress*

In order to analyze further whether Put level is related to ABA, NOX-dependent $O_2^{\cdot-}$ and Ca^{2+}, we investigated the effects of T (an inhibitor of ABA biosynthesis), three inhibitors (DPI, I and P) of NOX, EGTA (Ca^{2+} chelator), and $LaCl_3$ and verapamil (Ca^{2+} channel blockers) on Put content in wheat seedling leaves under osmotic stress. From figure 5, it could be showed that Put content increased markedly under osmotic stress. However, the increase induced by osmotic stress was inhibited by T treatment (from 173 to 121 nmol g^{-1} FW), which suggested that ABA might modulate Put biosynthesis. All of the treatments with three inhibitors (DPI, I and P) of PM-NOX activity inhibited the osmotic stress-induced increase of Put from 173 to 140 nmol g^{-1} FW or so, which indicated that Put biosynthesis might be modulated by PM-NADPH dependent $O_2^{\cdot-}$ production. The treatments with EGTA, La^{3+} and verapamil inhibited the increase of Put content from 173 to 125 nmol g^{-1} FW or so (figure 5), which suggested that Ca^{2+} might modulate Put biosynthesis.

4. Discussion

4.1 *Relationship between ABA and Put in wheat seedling leaves under osmotic stress*

The response of plant to water stress has been extensively investigated (Butt *et al*. 2015; Du *et al*. 2018). ABA is a significant regulator of plant responses to water stress (Larkindale and Knight 2002). Although it has also been well verified that water stress can facilitate the accumulations of PAs (Liu *et al*. 2005a; Pál *et al*. 2018), it is not clear whether the changes in ABA contents are physiologically relevant to PAs. The study of Liu *et al.* (2005b) shows the production of PAs is enhanced by ABA in maize seedlings subjected to salt stress. In the present study, osmotic stress brought about marked increases in Put (figure 1) and ABA contents (figure 2) in wheat seedling leaves. The results from experiments with T, an inhibitor of ABA biosynthesis (figure 1), suggested that Put content is regulated by ABA. However, the results from experiments with D-Arg (figure 2) suggested that ABA is not regulated by Put in wheat seedling leaves under osmotic stress. Taking the notions above together, it could be concluded that Put might function downstream of ABA in the osmotic stress-induced ABA signal transduction event in plants.

4.2 *Relationship between PM-NOX-dependent $O_2^{\cdot-}$ production and Put in wheat seedling leaves under osmotic stress*

In the osmotic stress-induced ABA signal transduction pathway, ABA could activate the PM-NOX to form $O_2^{\cdot-}$, then $O_2^{\cdot-}$ is turned into H_2O_2 by dismutation (Pei *et al*. 2000; Murata *et al*. 2001). So, it is obvious that PM-NOX-dependent $O_2^{\cdot-}$ is downstream of ABA in the ABA signaling. Then, whether Put accumulation induced by osmotic stress was associated to PM-NOX-dependent $O_2^{\cdot-}$? In the present research, PEG treatment led to the increases in Put content and the rate of PM-NOX-dependent $O_2^{\cdot-}$ production. With D-Arg treatment, it could be showed that with the decrease of Put content (figure 1), the rate of PM-NOX-dependent $O_2^{\cdot-}$ production were also reduced accordingly (figure 3). Furthermore, exogenous Put reversed the inhibitory effects of D-Arg. These findings suggested that Put might modulate the of PM-NOX activity and the rate of PM-NOX-dependent $O_2^{\cdot-}$ production. Put might up-regulate the activity of PM-NOX and function upstream of oxidation production in osmotic stress-induced ABA signaling. Then, whether PM-NOX-dependent $O_2^{\cdot-}$ could regulate Put content? To explore the problem, three treatments with inhibitors (DPI, I and P) of PM-NOX activity (Murphy and Auh 1996) were carried out in the research. The results (figure 5) with three inhibitors indicated that PM-NOX-dependent $O_2^{\cdot-}$ production modulated Put biosynthesis. Taking together the notion mentioned above that Put might modulate the PM-NOX-dependent $O_2^{\cdot-}$ production, it could be concluded that a cross-talk between Put and PM-NOX-dependent $O_2^{\cdot-}$ was involved in the ABA signaling pathway induced by PEG osmotic stress.

4.3 *Relationship between Ca^{2+} and Put in wheat seedling leaves under osmotic stress*

Since that a cross-talk between PM-NOX-dependent $O_2^{\cdot-}$ and Ca^{2+} is involved in osmotic stress-induced ABA signaling pathway (Chen and Li 2001; Yang and Poovaiah 2002; Jiang and Zhang 2003; Kreslavski *et al*. 2012) and $O_2^{\cdot-}$ production triggers Ca^{2+} influx and the increase in cytosolic Ca^{2+}, taking together the above notion that Put modulated PM-NOX-dependent $O_2^{\cdot-}$ production, it could be concluded that Put might up-regulated cytosolic Ca^{2+} level in the signaling pathway. However, whether Ca^{2+} modulates Put level remains unknown. Therefore, three treatments with Ca^{2+} chelator EGTA (Blume *et al*. 2000), Ca^{2+} channel blockers, $LaCl_3$ and V (Pei *et al*. 2000) were carried out in the research. The previous results (Jiang and Zhang 2003) verified that EGTA, $LaCl_3$ and V could reduce cytosolic Ca^{2+} level. Our present study showed that EGTA, $LaCl_3$ and V significantly reduced the Put content (figure 5). The same results obtained with the three different inhibitors suggested that cytosolic Ca^{2+} might up-regulate Put biosynthesis in the signaling pathway. From the suggestion and the notion mentioned above that Put up-regulated the cytosolic Ca^{2+} level, it could be concluded that a cross-talk between Put and cytosolic Ca^{2+} was also involved in osmotic stress-induced ABA signaling pathway.

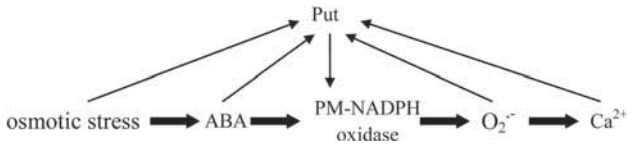

Figure 6. The involvement of Put in osmotic stress-induced ABA signaling and the cross-talks between Put, $O_2^{\cdot-}$ and Ca^{2+}. The arrow indicates the positive regulation (The coarse arrows present the results that have been already documented, and the thin arrows present the results in the present research).

5. Conclusion

The research first indicated that osmotic stress-induced Put was involved in the stress-induced ABA signaling pathway (osmotic stress→ABA→PM-NOX→$O_2^{\cdot-}$→H_2O_2→Ca^{2+}….) in wheat seedling leaves and there were cross-talks between Put, $O_2^{\cdot-}$ originated from PM-NOX and Ca^{2+} in the transduction pathway (figure 6).

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Grant No.: 31271627) and Science and Technology Project of Henan Province (Grant No.: 192102110135).

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Corresponding editor: MANCHIKATLA VENKAT RAJAM