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Transcriptional co-regulation of plasma membrane H^+-ATPase and NADPH oxidase during root growth

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ABSTRACT

During root growth, plasma membrane NADPH oxidase (NOX) and PM H^+-ATPase function cooperatively to maintain the membrane electrical balance while mediating cell growth through wall relaxation. A threshold $[Ca^{+2}]_{cyt}$ and H_2O_2 mediate the feed-forward loop between these two enzymes in *Vigna radiata* seedlings. The apoplastic superoxide ($O_2^{\bullet-}$) being produced by NOX is subsequently dismutated to H_2O_2 either spontaneously or by the activity of apoplastic Cu/Zn superoxide dismutase (SOD) enzyme. Since SOD utilises apoplastic H^+ (extruded from cytosol by PM H^+-ATPase) and $O_2^{\bullet-}$ as substrates, its functioning is inevitably depending on PM H^+-ATPase and NOX activities. Further conversion of H_2O_2 by class III peroxidase (Prx) to hydroxyl radical ($^{\bullet}OH$) is instrumental for wall polysaccharide cleavage and cell wall relaxation. Thus, SOD and Prx activities appear to be co-ordinated with the upstream interplay between NOX and PM H^+-ATPase in the regulatory network for root growth (Majumdar and Kar, 2019, J Plant Physiol 232: 248–256). In the present investigation, possible co-regulation among NOX and PM H^+-ATPase, at gene expression level, has been studied during root growth of *V. radiata* seedlings using quantitative real-time PCR (qRT-PCR). It is evident that a transcriptional co-regulation exists between the two enzymes and inhibition of any one enzyme or removal of its product represses gene expression of the other. As observed for their activities, H_2O_2 and Ca^{+2} apparently regulate expression of both NOX and PM H^+-ATPase genes and the probable mechanism is discussed.

1. Introduction

Continuous root growth enables plants to cope up with the increasing demand of water and nutrients. Although rapid cell division is a pre-requisite of root growth, majority of the enhancement in volume is obtained from cell elongation/expansion which depends on the extensibility property of the cell wall. The irreversible expansion of plant cells depends largely on cell wall loosening (Hager, 2003; Cosgrove, 2016a, 2016b). Plasma membrane (PM) H^+-ATPase is an electrogenic proton pump and functions as a key component in the process of cellular growth in plants. It is responsible, apart from many other functions (e.g. building up of turgor pressure), for acidification of apoplast (in an auxin-inducible manner) and activation of expansins and enzymes that are involved in cell wall loosening (Hager, 2003; Janicka-Russak, 2011; Falhof et al., 2016). Indispensible involvement of PM H^+-ATPase is well reported in root growth (Janicka-Russak, 2011; Janicka-Russak et al., 2012; Majumdar and Kar, 2018). On the other hand, studies through last few decades have identified apoplastic reactive oxygen species (ROS) to play pivotal roles in cell wall relaxation process (Liszkay et al., 2004). Apoplastic ROS circuit is initiated with NADPH oxidase [NOX; *R*espiratory *B*urst *O*xidase *H*omologs (RBOH)]-mediated production of superoxide ($O_2^{\bullet-}$) by one electron reduction of O_2, which is subsequently dismutated to hydrogen peroxide (H_2O_2) either spontaneously or by apoplastic Cu/Zn superoxide dismutase (SOD). It is reported that SOD utilises both apoplastic H^+ (extruded from cytosol by PM H^+-ATPase) and $O_2^{\bullet-}$ as substrates to produce H_2O_2 thereby being dependent on the activities of PM H^+-ATPase and NOX (Majumdar and Kar, 2019). Conversion of H_2O_2 by class III peroxidase (Prx) to hydroxyl radical ($^{\bullet}OH$) is instrumental for non-enzymatic cleavage of wall polysaccharides which results in enhancement of plastic extensibility of cell wall (Liszkay et al., 2004; Cosio and Dunand, 2009; Airianah et al., 2016).

Interestingly, a putative feed-forward loop between NOX and PM H^+-

Abbreviations: CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DPI, diphenyleneiodonium chloride; NOX, NADPH oxidase; PG, propyl gallate; Prx, class III peroxidase; SOD, superoxide dismutase; SNP, Sodium nitroprusside; Vanadate, sodium ortho-vanadate.

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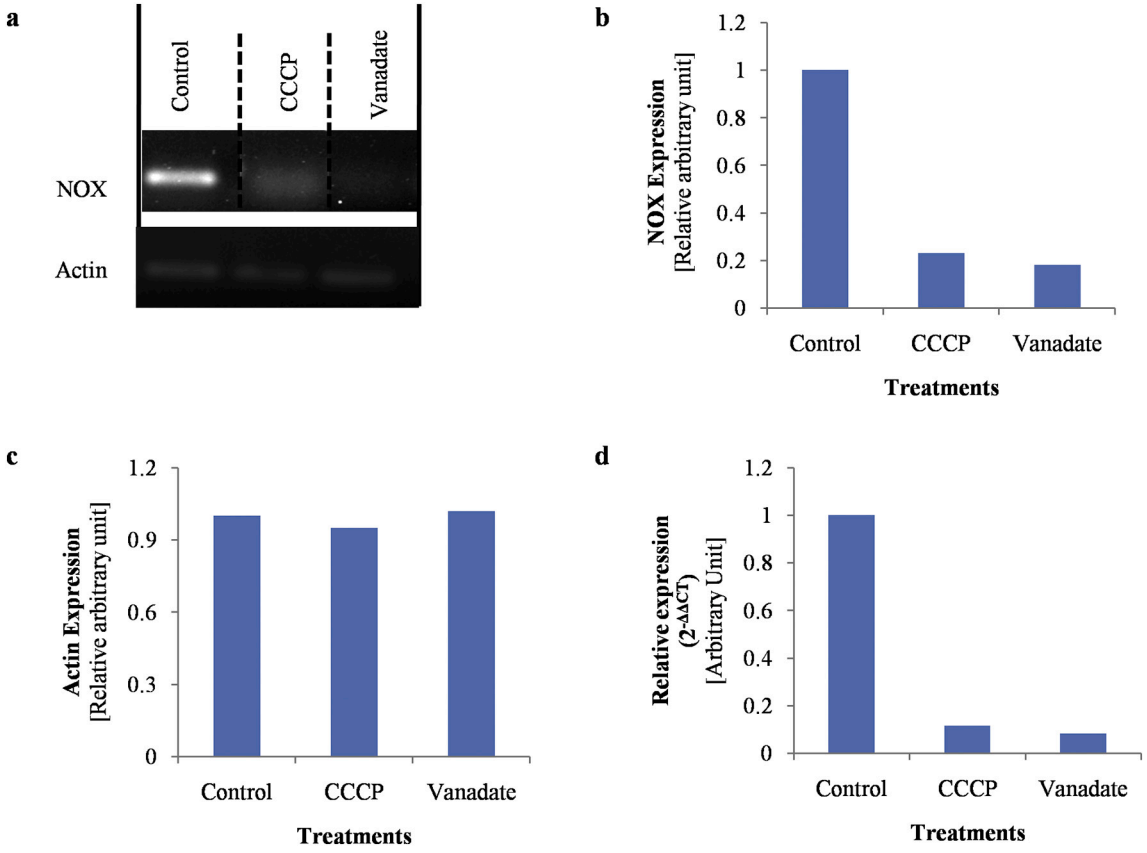

Fig. 1. Analyses of NADPH oxidase gene expression based on semi-quantitative and quantitative Real-time (qRT) PCR in roots of *Vigna radiata* seedlings. (a) Semi-quantitative reverse transcription PCR-derived amplicons of *V. radiata* NOX and *V. radiata* Actin genes from CCCP, vanadate-treated and control sets resolved in 1.5% agarose gel. (b) Relative band intensities of *V. radiata* NOX gene under different treatments in agarose gel. (c) Relative band intensities of *V. radiata* Actin gene under different treatments in agarose gel. (d) qRT-PCR analysis of *V. radiata* NOX gene demonstrating relative expression of the gene in *V. radiata* roots treated with CCCP and Vanadate along with control sets.

ATPase has recently been identified that integrates the apparent involvement of both PM H^+-ATPase-induced H^+ efflux and NOX-generated ROS in the cell wall loosening during root growth (Majumdar and Kar, 2018). The enzymes' activities have been found to be functionally synchronized during chloroplast avoidance movement too (Majumdar and Kar, 2020). Inhibition of either NOX or PM H^+-ATPase results in reduced activity of the other. Calcium (Ca^{+2}) functions as a potent mediator of the loop. Further, H_2O_2 has been found to promote both the enzymes which may be attained either directly or through acceleration of Ca^{+2}-entry into the cell across PM facilitating the building up of threshold $[Ca^{+2}]_{cyt}$ concentration (Li et al., 2011; Majumdar and Kar, 2018). Moreover, the interplay between NOX and PM H^+-ATPase has been documented to regulate the (downstream) activity of SOD and Prx as well, thereby depicting a probable synchronized functioning of the four enzymes during root growth (Majumdar and Kar, 2019).

Based on the harmonized functioning of PM H^+-ATPase and NOX, an intriguing question arises that whether such reciprocal regulation involves the expression of the respective genes too. Reports available in this regard are scanty and most of them are inconclusive. In the present investigation attempts have been made to recognize and comprehend the transcriptional co-regulation between PM H^+-ATPase and NOX during root growth of *Vigna radiata* (L.) Wilczek. A working model depicting the probable functional mechanism, involving the role of Ca^{+2} and other signalling agents, has been proposed.

2. Materials and methods

2.1. Plant materials and growth conditions

Surface sterilized seeds of *Vigna radiata* (L.) Wilczek were germinated for 12 h in dark on moistened Whatman No. 1 filter paper at 30 ± 2 °C. After germination, the seeds were transferred to different test solutions (treatments) or distilled H_2O (control) and incubated for 48 h in dark at the same temperature range. Roots of the 48 h grown seedlings were harvested and utilized for assessment.

2.2. Total RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was extracted from the root tissues following manufacturer's specifications using the RNeasy mini Plant kit (Qiagen). RNA yield was determined using NanoDrop Spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Scientific). *Vigna radiata* gene sequences were obtained from NCBI and gene-specific primers were designed or obtained from literature and cDNA were used as template in PCR reactions. ChemiDoc XRS$^+$ imaging system (Bio-Rad) was used for visualizations of bands and capturing images. Band intensities of the agarose gels were measured using ImageJ software. The QuantStudio 3 RealTime PCR system (Applied Biosystems) was used to perform qRT-PCR. Relative abundance of transcripts of respective genes was determined using Actin gene as reference. The transcriptional levels of NOX and PM H^+-ATPase genes were normalized against Actin and analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Among the several *Vigna radiata* mRNA sequences available in NCBI GenBank

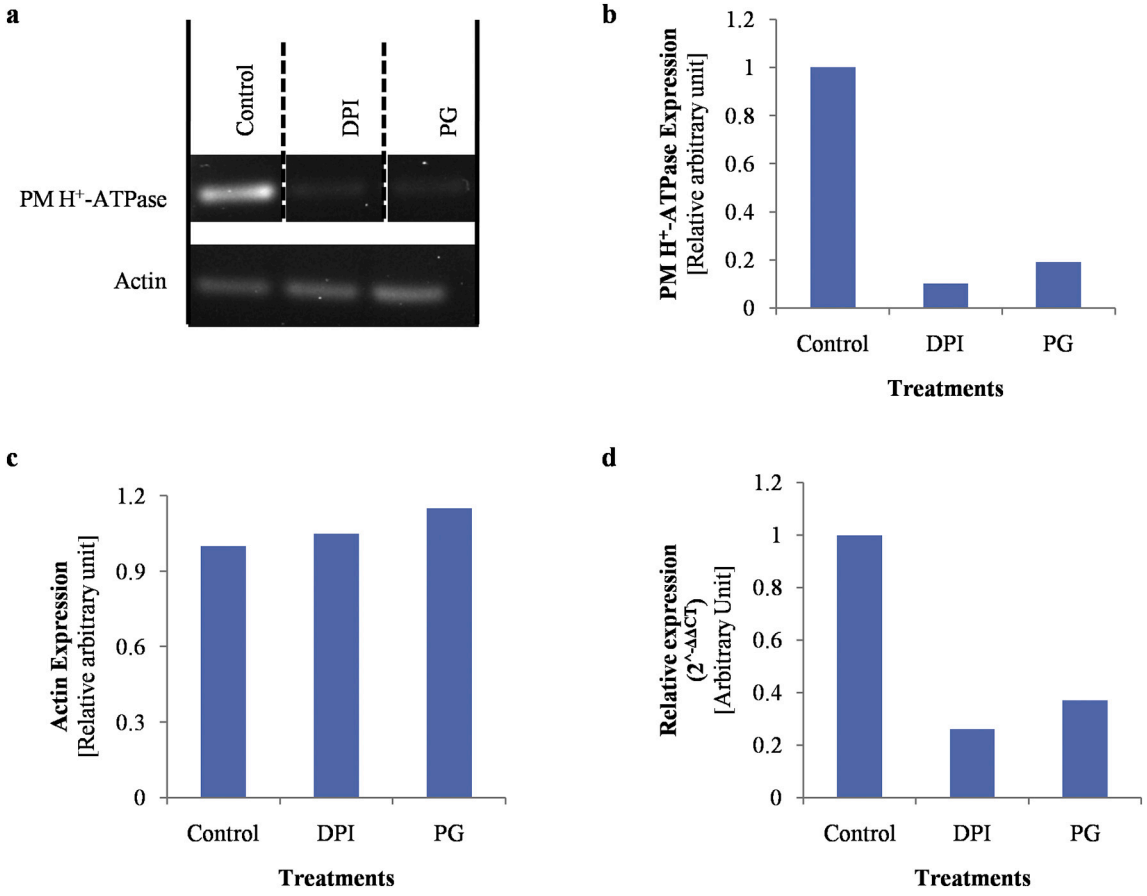

Fig. 2. Analyses of PM H^+-ATPase gene expression based on semi-quantitative and quantitative Real-time (qRT) PCR in roots of *Vigna radiata* seedlings. (a) Semi-quantitative reverse transcription PCR derived amplicons of *V. radiata* PM H^+-ATPase 4 and *V. radiata* Actin genes from DPI, PG-treated and control sets resolved in 1.5% agarose gel. (b) Relative band intensities of *V. radiata* PM H^+-ATPase 4 gene under different treatments in agarose gel. (c) Relative band intensities of *V. radiata* Actin gene under different treatments in agarose gel. (d) qRT-PCR analysis of *V. radiata* PM H^+-ATPase 4 gene demonstrating relative expression of the gene in *V. radiata* roots treated with DPI and PG along with control sets.

database, *V. radiata* PM H^+-ATPase 4 (transcript variant X1) and *V. radiata* RBOHC (transcript variant X2) (corresponding to PM H^+-ATPase and NOX genes, respectively) were selected for experiments in the present investigation. The selected mRNAs have conserved analogous sequences with *Arabidopsis thaliana* AHA2 and RBOHC mRNAs, respectively. Both AHA2 and RBOHC have been reported to express almost exclusively in roots. Details of the sequences (as available in NCBI GenBank database) along with the primers used for PCR are mentioned below:

***V. radiata* Actin** [Accession: XM_014658078; Version: XM_014658078.2; PREDICTED: *Vigna radiata* var. *radiata* actin (LOC106771984), transcript variant X1, mRNA; Available from: https://www.ncbi.nlm.nih.gov/nuccore/XM_014658078.2].

Forward: 5′- CACAGAAGCACCACTCAATCC - 3′.

Reverse: 5′ - CCATCACCAGAGTCCAGAACA - 3′.

***V. radiata* NOX** [Accession: XM_014654036, Version: XM_014654036.2; PREDICTED: *Vigna radiata* var. *radiata* respiratory burst oxidase homolog protein C (LOC106768745), transcript variant X2, mRNA; Available from: https://www.ncbi.nlm.nih.gov/nuccore/XM_014654036.2].

Forward: 5′ – CTAGCCCTGTGGATTGGTGT – 3’.

Reverse: 5′ – CCTTAGCCAGGTGATGGTGT – 3′.

***V. radiata* PM H^+-ATPase 4** [Accession No.: XM_014645401, Version: XM_014645401.2; PREDICTED: *Vigna radiata var. radiata* plasma membrane ATPase 4 (LOC106761834), transcript variant X1, mRNA; Available from: https://www.ncbi.nlm.nih.gov/nuccore/XM_014645401.2].

Forward: 5′ – AAGAGGCTGCAAGAGAGGAA – 3’.

Reverse: 5′ – GACCAGGTTCAGTGAGGACAA – 3′.

3. Results

To assess whether treatments with PM H^+-ATPase inhibitor [sodium ortho-vanadate (Na_3VO_4); 100 μM] and protonophore [carbonyl cyanide chlorophenylhydrazone (CCCP); 50 μM] affect gene expression of *V. radiata* NOX, semi-quantitative and quantitative real-time PCR (qRT-PCR) analyses were performed with total RNA extracted from roots of 2-days grown seedlings (Fig. 1). The semi-quantitative PCR derived amplicons were resolved in 1.5% agarose gels (Fig. 1a). The level of transcript of *V. radiata* NOX gene was greatly reduced, as indicated by the band intensities, under both CCCP [A.U. (expression arbitrary unit) 0.27] and vanadate (A.U. 0.12) treatments compared to control (A.U. 1.00) (Fig. 1b). The expression of reference Actin gene was similar under the treatments [CCCP: A.U. 0.95; Vanadate: A.U. 1.02] and control set (A.U. 1.00) (Fig. 1c). This result was further corroborated by qRT-PCR assay, which showed a sharp decline in relative expression of *V. radiata* NOX gene under both the treatments. Thus, the transcript level of the gene was much lower in CCCP (A.U. 0.23) and Vanadate (A.U. 0.29) treated plants relative to the control (A.U. 1.00) plants (Fig. 1d). This clearly indicates the involvement of PM H^+-ATPase activity and a stable cross-PM H^+ gradient in regulation of *V. radiata* NOX gene expression.

Conversely, semi-quantitative PCR analyses were performed to determine the effect, if any, of NOX inhibitor [diphenyleneiodonium

chloride (DPI); 10 μM] and ROS scavenger [propyl gallate (PG); 50 μM] on *V. radiata* PM H^+-ATPase 4 gene expression (Fig. 2a). Relative band intensities (in 1.5% agarose gel) were much lower for the lanes corresponding to the treatments [DPI: A.U. 0.1; PG: A.U. 0.19] than the control lane [A.U. 1.00] (Fig. 2b). The expression of Actin gene remained unchanged under the treatments [DPI: A.U. 1.05; PG: A.U. 1.15] relative to control set (A.U. 1.00) (Fig. 2c). Validation of the data was obtained again from qRT-PCR analyses which confirmed repression of *V. radiata* PM H^+-ATPase 4 gene expression under the treatment of DPI and PG. Both the treatments restricted transcript levels to much lower values [DPI: A.U. 0.26; PG: A.U. 0.37] relative to control [A.U. 1.00] (Fig. 2d). Thus, inhibition of NOX or scavenging of ROS [being produced in a ROS cascade initiated with NOX activity] evidently resulted in down regulation of *V. radiata* PM H^+-ATPase 4 gene expression.

4. Discussion

Being the initiator of apoplastic ROS cascade, NOX plays pivotal roles in root growth. While regulation of NOX activity is dependent on several factors viz. Ca^{+2}-binding, phosphorylation etc., expression of NOX has been reported to be regulated by MAPK (mitogen-activated protein kinase) cascades. It has been observed that phosphorylation of WRKY transcription factors (large family of zinc-finger type TFs) by MAPKs enables the protein to bind to the W-box DNA *cis*-element (5'-TTGACC/T-3'), located in the NOX/*RBOHB* gene promoter, and results in positive regulation of expression of RBOHB gene in *Nicotiana benthamiana* (Yoshioka et al., 2016; Hu et al., 2020). Adachi et al. (2015) have reported that silencing of WRKY 7/8/9/11 genes significantly reduced RBOHB gene expression and thus impaired "ROS burst" during INF1-triggered PTI (pattern-triggered immunity) and R3a/AVR3a-triggered ETI (effector-triggered immunity). Interestingly, MAPK cascades are ROS-responsive and H_2O_2 can activate MAPK cascades e.g. MPK3, MPK4, MPK6 (Liu and He, 2017) which would promote phosphorylation (and activation) of WRKY TFs and up-regulation of target genes' expression. Thus, it is evident that H_2O_2 is involved in regulation of NOX expression too. It has recently been reported that during root growth, PM H^+-ATPase and NOX activities are synchronized and they function in a continuous co-ordinated feed-forward loop which enables steady production of apoplastic H_2O_2 by SOD (Majumdar and Kar, 2018, 2019). The de novo generated apoplastic H_2O_2 then crosses PM through aquaporins (Mubarakshina and Ivanov, 2010; Bienert and Chaumont, 2014) and functions as a signalling molecule. The apoplastic "ROS signal" is transmitted to nucleus via several agents viz. Ca^{+2}, MAPKs and different transcription factors (TFs) (Galon et al., 2010; Shapiguzov et al., 2012) and essentially results in modulation of gene expression. Concomitantly, inhibition of PM H^+-ATPase (by Vanadate) and quenching of cross-PM H^+ gradient (by CCCP) disrupts the NOX-PM H^+-ATPase feed-forward loop and reduces H_2O_2 production (Majumdar and Kar, 2018). This depletion in H_2O_2 level inhibits activation of MAPK cascade and phosphorylation of WRKY TFs which eventually results into down-regulation of NOX expression (Fig. 1a, b, d). Confirmation of the hypothesis may be obtained from Dang et al. (2019) where the authors have demonstrated existence of a positive feedback loop between H_2O_2 accumulation and promotion of WRKY41 gene expression. Moreover, overexpression of WRKY41 gene resulted in significant up-regulation of NOX (RBOH C/D/E and F) genes.

On the other hand, expression and activity of PM H^+-ATPase are tightly regulated by diverse mechanisms which involve participation of different signalling agents (Janicka-Russak, 2011; Falhof et al., 2016). Phytohormones e.g. auxin have often been found to modulate the enzyme's expression during several plant processes (Frías et al., 1996). It has already been documented that ROS (especially H_2O_2) promote expression of genes by interacting with transcription factors whereas scavenging of ROS stalls gene expression (Foyer and Noctor, 2005; Volkov et al., 2006; Liu et al., 2012). Consequently, Janicka-Russak et al. (2012) have reported that treatment with exogenous H_2O_2 significantly enhanced the transcript levels of PM H^+-ATPase genes (*Cs*HA4, *Cs*HA8 and *Cs*HA9) in *Cucumis sativus* roots. In the present study, it has been observed that abolition of apoplastic ROS cascade by inhibiting NOX (using DPI) and scavenging ROS (by PG) inhibits PM H^+-ATPase gene expression (Fig. 2a, b, d). This conforms to earlier studies that DPI could significantly reduce PM H^+-ATPase gene expression even under the treatment of NaCl (promotes PM H^+-ATPase activity) and sodium nitroprusside (SNP; nitric oxide donor, promotes PM H^+-ATPase activity) (Zhang et al., 2007). Inhibition of NOX disrupts the NOX-PM H^+-ATPase feed forward loop resulting in diminution of H_2O_2 production (Majumdar and Kar, 2018) and PG scavenges the available H_2O_2 altogether. Corroborating to earlier reports, the resultant diminished H_2O_2 production leads to down regulation of PM H^+-ATPase gene expression. However, the mechanism of H_2O_2-induced PM H^+-ATPase gene expression has not been investigated thoroughly and the question remains mostly unanswered. Interestingly, a recent study has identified W-box sequence in the promoter region of PM H^+-ATPase gene *Pe*HA1 in *Populus euphratica* (Yao et al., 2020). The authors have reported that PeHA1 is a target gene of WRKY protein and the gene's expression was activated by binding of WRKY to W-box whereas silencing of PeWRKY gene down-regulated *Pe*HA1 expression. As H_2O_2 activates WRKY TFs by phosphorylation through stimulation of MAPK cascade, PM H^+-ATPase gene expression may also be regulated by H_2O_2 in a similar manner as hypothesized for NOX expression (through MAPK cascades and WRKY TFs).

Ca^{+2} is intrinsically involved with the regulation of PM H^+-ATPase and NOX activities (Gilroy et al., 2014; Kurusu et al., 2015; Majumdar and Kar, 2018). While Ca^{+2} regulates PM H^+-ATPase activity by phosphorylating specific amino acids e.g. Thr947, Thr955 through different CDPKs (Ca^{+2}-dependent protein kinases), it directly binds to the N-terminal EF hand motifs of NOX and activates the enzyme. CDPK-dependent phosphorylation is also necessary for NOX stimulation. Interestingly, development of threshold $[Ca^{+2}]_{cyt}$ concentration is necessary for such regulation and it depends on both the enzymes (Michelet and Boutry, 1995; Foreman et al., 2003; Demidchik et al., 2007). PM H^+-ATPase induces apoplastic acidification resulting into hyperpolarization of the membrane and activates HACCs (hyperpolarization activated calcium channels) that allows Ca^{+2} entry into the cytosol. Similarly, it is also documented that ROS stimulate inward-rectifying Ca^{+2} permeable channels in PM (Demidchik, 2018). However, apart from its role in activation of enzymes at post-translational stage, pivotal roles of Ca^{+2} in plants' gene expressions are also being revealed increasingly (Liu et al., 2020). Alteration in gene expression may be achieved through direct binding of Ca^{+2}-CaM (calmodulin) complex to transcription factors or via regulation of CDPKs and phosphatases in a dose-dependent manner (Kim et al., 2009; Galon et al., 2010). It has been reported that CaM binds at the conserved N-terminal C-motif of WRKY proteins in a Ca^{+2}-dependent manner and activate the TFs (Park et al., 2005; Galon et al., 2010; Rushton et al., 2010). Thus, Ca^{+2} apparently regulates expression of both PM H^+-ATPase and NOX genes.

.

5. Conclusion

A functional feed-forward loop exists between PM H^+-ATPase and NOX which is mediated by H_2O_2 and Ca^{+2} (Janicka-Russak, 2011; Li et al., 2011; Majumdar and Kar, 2018). However, apparent modulations of activities of both the enzymes are partly derived from altered expression of the respective genes (that essentially attune the available enzyme proteins). Co-ordinated functioning of PM H^+-ATPase and NOX produces H_2O_2 which stimulates expression of both the genes through MAPK cascades and WRKY transcription factors. Ca^{+2} regulates the activation of WRKY TFs and thereby influences the expression of PM H^+-ATPase and NOX genes. However, the establishment of threshold

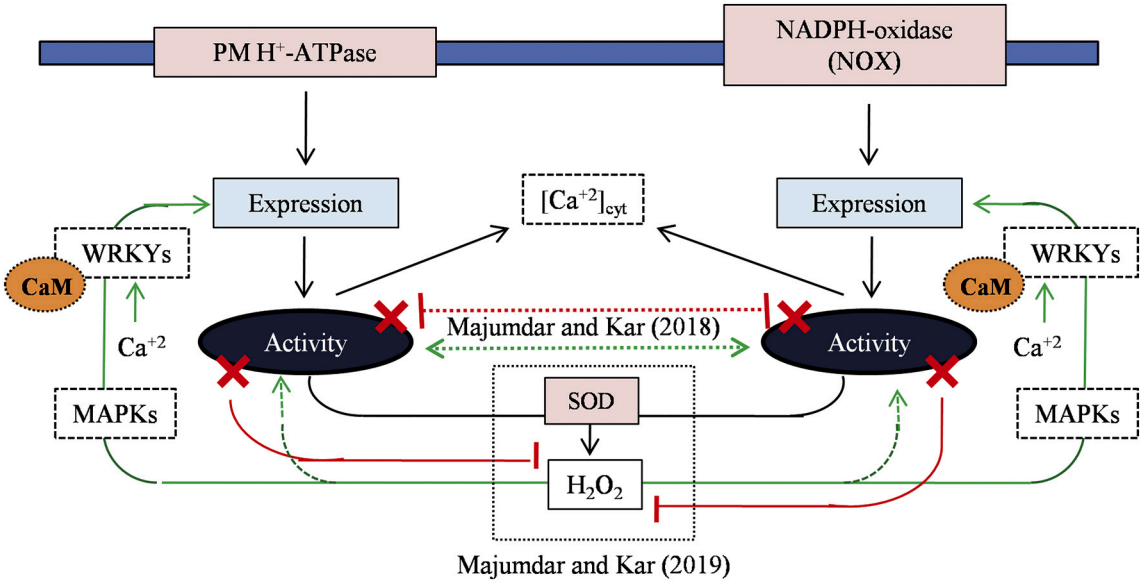

Fig. 3. A possible working model depicting the transcriptional co-regulation of PM H^+-ATPase and NOX. As a result of the functional synchronization of the enzymes, H_2O_2 is produced continuously (through the activity of SOD) during root growth and it can promote activities of both PM H^+-ATPase and NOX (Majumdar and Kar, 2018, 2019). Moreover, it can also regulate expression of both the genes through MAPK cascades and WRKY transcription factors. Threshold $[Ca^{+2}]_{cyt}$, which is built up depending on the two enzymes, modulate the activation of WRKY TFs by facilitating binding of CaM to a conserved N-terminal C-motif. Thus, inhibition of any one enzyme or removal of its product during root growth drastically reduces H_2O_2 production and impedes development of threshold $[Ca^{+2}]_{cyt}$ which leads to down-regulation of gene expression and inhibition of activity of both the enzymes.

$[Ca^{+2}]_{cyt}$ concentration depends on both the enzymes' activities. As inhibition of any one enzyme or removal of its product down-regulates gene expression of the other, it appears to be rational and justifiable to hypothesize that expression of NOX and PM H^+-ATPase genes are co-regulated during root growth of *V. radiata* (L.) Wilczek seedlings by H_2O_2 and Ca^{+2} through MAPKs and distinct TFs like WRKY (Fig. 3). Further in-depth studies are required to substantiate this proposition.

Author contribution statement

RKK visualized the study. AM and RKK conceptualized the work. AM performed the investigation. AM and RKK wrote the article.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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