University of São Paulo
“Luiz de Queiroz” College of Agriculture

The cell wall is crucial for cellular sensitivity to low pH: the role of class III peroxidases and ethylene in cell death in *Arabidopsis thaliana* roots

Jonathas Pereira das Graças

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

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Jonathas Pereira das Graças
Biologist

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Advisor:
Prof. Dr. **LÁZARO EUSTÁQUIO PEREIRA PERES**

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RESUMO

A parede celular é crucial para a sensibilidade celular ao baixo pH: o papel de peroxidases de classe III e etileno na morte celular em raízes de *Arabidopsis thaliana*

Evidencias recentes sugerem que a parede celular é um alvo direto do estresse por baixo pH em raízes. Estresse severo por baixo pH rapidamente causa a morte de células do ápice radicular, onde a parede é altamente dinâmica. Nossa hipótese é de que nessas células, o baixo pH cause mudanças na parede celular, como afrouxamento excessivo. Assim, a pressão de turgor sobre a parede deve ser necessária para causar danos que levam à morte das células. Neste trabalho, nós investigamos o papel da parede celular no estresse por baixo pH e na consequente morte de células radiculares. Além disso, tambem foi investigado o papel de peroxidases de classe III e sinalização por etileno, que promovem mudanças na parede celular as quais podem gerar sensibilidade diferenciada a baixo pH. Plântulas de *Arabidopsis thaliana* e mutantes no background de Col-0 foram crescidas em meio contendo ágar (0.8%) e metade da concentração dos nutirentes do meio de Hoagland. Plântulas com 5 dias de idade foram expostas a baixo pH em uma solução composta por 0.5 mM de $CaCl_2$ e 0.6 mM de tampão Homopipes. O tratamento de raízes a pH 4.6 causou morte em células da zona de transição (TZ) e zona meristemática (MZ). Entretanto, a morte celular foi negligível quando as plantas foram tratadas a pH 4.6 simultaneamente com a diminuição da tensão na parede celular, através de solução com potencial de - 0.37 MPa. Além disso, um choque repentino na pressão de tugor por intermédio de tratamento hiposmótico (HO) causou morte celular a pH 5.8 na TZ. A morte celular foi acelerada quando HO foi realizado em uma solução a baixo pH. A morte celular foi reduzida no mutante *wak-1* exposto a baixo pH. WAK-1 é um receptor de parede que atua no sistema de monitoramento de integridade da parede celular. A morte das células provavelmente ocorreu por meio de morte celular programada. Juntos, esses dados trazem evidências que a parede celular é crucial para percepção do estresse causado por baixo pH e essa percepção possivelmente está envolvida em repostas que causam a morte celular. Nós examinamos dados publicados procurando por peroxidases classe III possivelmente envolvidas com a morte celular devido baixo pH. O gene codante para AtPRX62 foi induzido 8.37 vezes em raízes expostas a baixo pH. O mutante KO *atprx62* foi menos sensível a baixo pH que raízes de Col-0. O mRNA de AtPRX62 acumulou-se na mesma zona de morte celular devido baixo pH em raízes de Col-0. Isso sugere que a atividade de AtPRX62 está relacionada com a morte celular devido baixo pH. Além disso, o pré-tratamento com etileno induziu tolerância de raízes à exposição subsequente a baixo pH. Esta indução foi dependente de sinalização via ETR1. No conjunto, nós mostramos que um estresse causado na parede celular pelo baixo pH causa a morte celular. Essa morte é em parte devido a atividade de AtPRX62 mas pode ser aliviada por etileno.

Palavras-chave: Acidez; Integridade de parede celular; Peroxidases de classe III; Etileno; Morte celular; Tensão de parede celular

ABSTRACT

The cell wall is crucial for cellular sensitivity to low pH: the role of class III peroxidases and ethylene in cell death in *Arabidopsis thaliana* roots

Evidence suggests that root cell walls are a target of low pH stress. Severe low pH stress causes cell death in the root tip. The walls of these cells are highly dynamic. Our hypothesis is that in these cells low pH causes stress in the cell wall due to excessive loosening. Thus, a certain level of turgor pressure should be required to cause cell death. Here, we aimed to investigate the role of the cell wall in low pH stress leading to cell death. We looked for the possible involvement of players such as class III peroxidases and ethylene signaling, which could promote changes in the cell wall and cause differential sensitivity to low pH. *Arabidopsis thaliana* and mutants in the genetic background of Col-0 were grown in a medium containing agar (0.8%) and half the concentration of Hoagland's nutrient medium. Five-day-old seedlings were exposed to low pH in a solution composed of 0.5 mM $CaCl_2$ and 0.6 mM Homopipes buffer. Treatment of roots at pH 4.6 caused death of cells in the transition zone (TZ) and meristematic zone (MZ). However, cell death was negligible when plants were treated at pH 4.6 in an hyperosmotic solution (Ψs = -0.37 MPa), thereby decreasing cell wall tension. Also, an hypoosmotic treatment (HO) caused cell death at pH 5.8 in TZ. Cell death was accelerated when HO was performed in a low pH solution. The mutant of a cell wall integrity sensor protein, *wak-1*, displayed reduced cell death when exposed to low pH. Also, cell death seems to occur through a programmed cell death mechanism. Thus, low-pH induced cell death appears to be triggered by perception of cell wall stress. We examined published data to search for class III peroxidases possibly involved in cell death due to low pH. The gene for AtPrx62 is induced 8.37-fold in low pH exposed roots. The *atprx62* KO mutant was less sensitive to low pH than Col-0 roots. The mRNA of *AtPRX62* accumulated in the same zone that cell death occurred due to low pH. This strongly suggests that AtPRX62 is positive regulator of low-pH induced cell death. Also, ethylene pretreatment induced subsequent tolerance of roots to low pH and this was dependent of its receptor ETR1. Together we show that a cell wall stress caused by low pH causes cell death. This death was in part due AtPRX62 activity and was also suppressed by ethylene.

Keywords: Acidity; Cell wall integrity; Class III peroxidases; Ethylene; Cell death; Cell wall tension

1. INTRODUCTION

Almost 40% of the world's agricultural areas are covered by acidic soils with a pH value below 5.0 (Vonuexkull and Mutert, 1995). In Brazil, there are about 500 million hectares of acid soils (Vitorello et al., 2005). These soils are characterized by low nutrient concentrations and solubilization of toxic forms of Al (Kochian, 1995). The combination of these factors is known as "acid soil syndrome" which results in inhibition of root development.

Many studies have focused on Al and soil nutrients. However, most of the toxic forms of Al to plants are only active at low pH. Thus, low pH has obvious implications for understanding of Al toxicity.

Indeed, low pH by itself can be toxic to the root system. The irreversible consequence of this toxicity is cell death that can happen within a short time of low pH exposure, such as half an hour (Koyama et al., 2001; Gracas et al., 2016).

Events induced by low pH in min have been previously described, such as changes in plasma membrane permeability and ion uptake (Bose et al., 2010). This can be crucial for nutrient acquisition and cellular homeostasis. However, it remains unclear whether such events are just merely effects of low pH, which within the time of exposure, might kill cells or might be part of a signaling that in ultimate is linked to the death of cells.

Evidence is emerging that the interaction between low pH and cell wall seems to be relevant for the sensitivity of cells to the stress (Bibikova et al., 1998; Monshausen et al., 2007; Horst et al., 2010; Lager et al., 2010; Gracas et al., 2016). The nature of this interaction and what this might change on cell wall is so far unknown.

Few works presented evidence that low pH can directly disturb cell wall integrity. The expansion of root hairs requires fine control of apoplast acidification. Expanding root hairs burst if maintained at pH 4.5 (Bibikova et al., 1998; Monshausen et al., 2007). However, root hairs that in which expansion had stopped did not burst at pH 4.5. One interpretation for this is that the root hairs probably burst due to cell wall failure caused by low-pH induced derangements, which are enhanced by turgor pressure. It is reasonable to suggest that others root cells can be affected by low pH in a similar mode, due to cell wall derangements.

It is known that root tip cells are the most sensitive cells to low pH (Koyama et al., 2001). A major challenge is to determine what special features of these cells cause this sensitivity to low pH. Indeed, these cells, which are situated in the MZ, TZ and EZ, possess remarkable differences concerning their development and responses to hormone signaling. One inevitable fact is that these cells, upon reaching the end of the EZ, will have become more than a hundred times larger than their initial size in the MZ (Verbelen et al., 2006). In order to accommodate this growth, the cell wall must be highly dynamic, with large changes in its properties over time. The potential for errors appears to be great.

Cells have a system to monitor cell wall integrity allowing correct growth and preventing disturbances (Somssich et al., 2016). This system involves several players as cell wall integrity sensors, hormones, ROS and the activity of enzymes that physically change the wall (Ringli, 2010).

CIII Prx can promote cross-linkage between polysaccharides and proteins, important for cell wall formation and for physical change in cell wall such as loosening or tightening (Passardi et al., 2005). These last two remarkable opposite activities in the same structure make peroxidases important players for rapid response to biotic or abiotic constraints upon the wall.

An increase of H_2O_2 due to peroxidase activity triggered by Al stress was linked to the death of root cells (Simonovicova et al., 2004). Most probably, an uncontrolled ROS production caused damages to the cells. After wounding in roots, CIII Prx increased its activity and there was increased production of ROS (Minibayeva et al., 2009; Minibayeva et al., 2015). This may be useful to change cell wall properties for plant defense or might have a direct action upon pathogen infection. Also wounding response may invoke cell death dependent on ROS and peroxidases (Minibayeva et al., 2015). Application of SHAM, an inhibitor of CIII Prx activity greatly increased cell death in tomato roots treated with low pH (Gracas et al., 2016). Hence, CIII Prx activity and ROS seems to be responsive to cell wall stresses.

Plants under Pi starvation quickly inhibits the root growth through the activation of two noncoupled pathways involving peroxidases that stiffen cell walls in TZ and this results in the arrest the growth (Balzergue et al., 2017). One CIII Prx isoform, AtPRX71, was related to ROS production due to treatment with isoxaben that disrupts cell wall integrity (Raggi et al., 2015). This ROS production was interpreted as linked to responses for coordinated decrease in cell expansion under stress.

CIII Prx utilizes ROS and fine controls its metabolism due to their dangerous potential to the cells. Whether peroxidase activity may be involved in cell death or if are part of plant tightening to constraints, these enzymes, are clearly involved on different stresses responses. The role of individual isoforms must be investigated in low pH stress

Ethylene is well known as abiotic stress response hormone (Raja et al., 2017) and for signaling responses upon cell wall in the whole plant. In roots, application of the ethylene precursor ACC rapidly inhibits cell elongation (Le et al., 2001). This control upon elongation is in part achieved by ethylene due to a transcriptional control of cell wall-related enzymes (Markakis et al., 2012). Among these enzymes are CIII Prx. These enzymes act upon cell wall changing its properties for stop of cell expansion. Thus, due its role in cell elongation and transcriptional control of peroxidases ethylene involvement in low pH response must be investigated

PCD is part of normal root development for events as xylogenesis, control of root cap cells and death of old root tissues (Bagniewska-Zadworna and Arasimowicz-Jelonek, 2016)). In roots, PCD is also induced by an increase in mitochondrial release of ROS induced by Al treatment (Huang et al., 2014). Al and low pH share commonalities but they might also have different targets in root cells. Hence, it would be discerning to investigate if the death of cells during low pH can be through a PCD mechanism.

Interesting, evolution has selected PCD as strategy for survival. Bacteria growing in colonies may face nutrient starvation. This threatens all individuals. A wave of PCD spreads through the colony, as if they were a single organism, reducing the population and allowing survival of the remaining avoiding nutrient competition (Engelberg-Kulka et al., 2006).

Since before the postulation of “acid growth theory” the interaction between low pH and cell wall is seem as relevant for understanding plant growth and development. Thus, understand the cell wall response due to low pH and determine players for this response is a central topic in plant biology. This work aimed to investigate the effect low pH toxicity in roots and its possible interaction with cell wall in the MZ, TZ and EZ.

2. CONCLUSION

We have provided evidence that, taken together, demonstrates that the cell wall has a crucial role in the death of root tip cells caused by low pH stress. A considerable part of the response to low pH appears to be due to the activity of class III peroxidases. Cell death also appeared to be partially dependent on ETR1 signaling and could be reversed with ethylene pretreatment.

Our first results showed that low pH increased cell wall extensibility in epidermal cells of the TZ. This event or some other cell wall instability due to low pH was required for the onset of cell death. Thus, the cell wall is a target of low pH stress. However, cell death did not appear to be a direct consequence of cell wall disorders, rather it appeared to be triggered by perception of cell wall integrity and activation of a signaling pathway leading to programmed cell death. Considering this, it is perhaps not surprising that gene expression responses to low pH resembled those in response to pathogens and their elicitors (Lager et al. 2010). Understanding the cell wall integrity system is a central theme in plant biology. Thus, knowledge of the dynamics of the cell wall under low pH may contribute to this field of research.

The investigation of players involved in low pH-induced cell death appears to be complex. Nonetheless, it was remarkable that a single isoform of peroxidase, AtPRX62, could have such a pronounced effect on low-pH induced cell death. Our results with ethylene, however, suggest that this peroxidase may be just one link, among others, of a signaling network for cell death. Elucidation of such a network, as well as the function and role of CIII Prx will require the combination of diverse approaches such as those discussed by Francoz et al. (2015).

Finally, cell death due to low pH as an orchestrated response through PCD opened several questions. It would be interesting to investigate, for example, if PCD confers any advantage for plant adaptation to an acidic environment.

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