Faculty of Agriculture, University of Poonch Rawalakot

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Jammu Kashmir Journal of Agriculture

ISSN: 2312-9344 (Online), 2313-1241 (Print)

https://jkjagri.com/index.php/journal

Short Communication

ARABIDOPSIS *ASCORBATE OXIDASE* MUTANT CHARACTERIZATION TO DETERMINE ITS PHYSIOLOGICAL ROLE IN PLANT DEVELOPMENT

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A B S T R A C T

The T-DNA insertional mutant assemblies of genes of *Arabidopsis thaliana* are making a significant contribution to research in plant biology since past two decades. A broad-spectrum of problem in plant biology intended at in-vivo gene characterization for various functions were being studied by these mutant alleles. The study was undertaken to appraise the regulatory role of *ascorbate oxidase* gene in plant development to see its role in reactive oxygen species. To evaluate the functional role of *ascorbate oxidase* a mutant lacking AO was compared to the wild-type. For this purpose, a salk-line homozygous for an insertion in the AO coding sequence was used and verified for the presence of the insertional mutagenesis. Much effort needs to complete this picture, and ultimately fulfill the present-day gap in our judgement of such potential instigator of plant responses.

Keywords: Salk-line; *Arabidopsis*; Ascorbate oxidase; T-DNA; Mutagenesis

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Article history
Received: June 14th, 2021
Revised: July 29th, 2021
Accepted: September 12th, 2021

INTRODUCTION

Reverse genetics approach like gene knockout or t-DNA insertion libraries of mutants is an important tool to study contribution of gene to physiological, cellular, tissues and organs phenotyping and genotyping. The characteristics of mutant genotype having different action of genes in vivo can be evaluated by comparing phenotypically with the wild-type parent genotypes. (O'Malley et al., 2015). Ascorbate oxidase (AO) is widespread in the plant kingdom, and is likely to have a role in some basic mechanisms of plant adaptation. The metabolism of ascorbate accomplishes a central role in the detoxification of extravagant ROS in cells, hence regulating the balance of cellular-redox, that control plant growth and development and ultimately cellular responses to environmental stress (Khanna-Chopra, 2012; Lai et al., 2012). The selective decrease in level of both AsA contents and oxygen in region of apoplast worked on one side by decreasing oxygen level thus limiting reactive oxygen formation (ROS), and on the other side by oxidizing AsA the primary apoplast antioxidant. The regulatory mechanisms of the apoplast redox state are the keys to persuade plant response toward biotic and abiotic stress (Pignocchi et al., 2003).

The functional role of ascorbate oxidase has never been fully explained so far, due to the difficulties in understanding the presence of an enzyme specifically

oxidizing ascorbate. The AO enzyme catalyzed a complex reaction, including safer oxygen reduction in water, without releasing ROS (hydrogen peroxide, superoxide anion), which result from partial O_2 reduction that can be beneficial for releasing extra oxygen under particular conditions. The role of AO is to modify the apoplastic redox state in such a way as to modify receptor activity and signal transduction to regulate defense and growth. The induction of AO transcription, leading to increased AO activity, would generate an 'inactive' or oxidized state in the apoplast, attenuating growth signal transduction processes and activating defense processes and *vice versa* (Farver et al., 1994; Pignocchi et al., 2003).

The validation of the physiological role of AO proved very challenging so far, as it perform multiple functions in plants. *ascorbate oxidase* (AO) is abundantly found in plants and fungi with relative great expression in developing tissues and young fruits (Hoegger et al., 2006; Kato and Esaka, 1996; Pignocchi et al., 2003; Sanmartin et al., 2007). Reactive oxygen species (ROS) are stared having signaling molecule which participated frequently in growth and development of plants, programmed-cell-death (PCD), and response to environmental stresses. The various enzymes of AsA antioxidant systems performed diverse functions; likewise *ascorbate peroxidase* (APX) enzyme work as a ROS-scavenging reducing hydrogen peroxide (H_2O_2) to water, whereas AO uses AsA as a substrate for reducing O2 to water as shown in figure 1 (Apel and Hirt, 2004; Li et al., 2007; Raven, 2000). All this information, suggesting in part a possible role of AO in signal perception/transduction, has been known but the function of this remarkable enzyme remained controversial (Garchery et al., 2013; Pignocchi et al., 2003; Yamamoto et al., 2005).

Because of the critical role of ROS equally as signaling molecules, it was important to study that mutants in case of ROS scavengers will have different phenotypic and genotypic expression in different lab imitating variation in growth conditions. This experiment goal involves the actualization of specific quantitative considerations to evaluate the functional role of AO, by comparing a mutant lacking AO and parent wild-type columbia and screening to identify T-DNA insertion in transformed *Arabidopsis* lines.

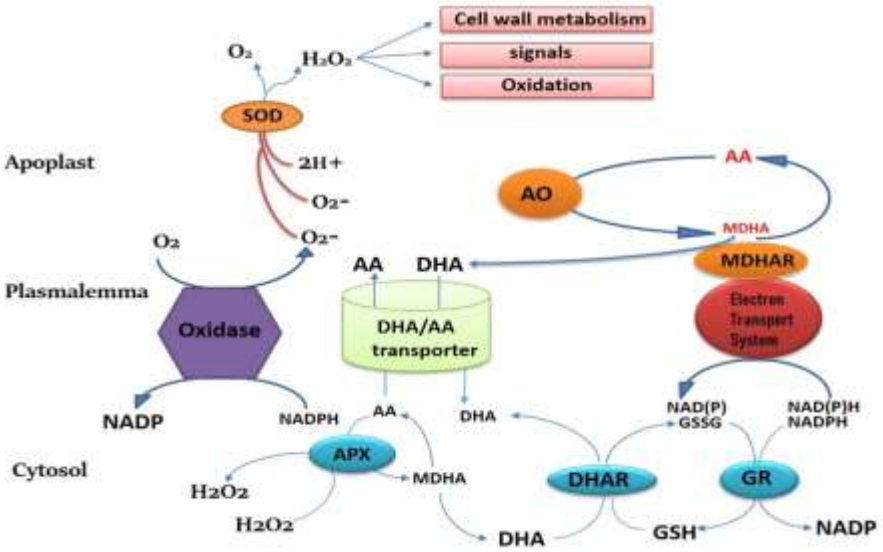

Figure 1. Schematic presentation of ascorbate metabolism in cell signaling Pathway. Origin of ROS main sources in plant cell. In such reactions beside single oxygen, formation of superoxide and hydrogen peroxide may take place in chloroplast by the process of reduction of molecular-oxygen (Quinlan et al., 2012). In apoplast ascorbic acid (AA), is the key and perhaps the single antioxidant buffer that became oxidized under such conditions. This apoplast *ascorbate oxidase* (AO) enzyme likewise regulated the reductional/oxidational (redox) states of ascorbates group in apoplastic region. The AO oxidative bursts will modify the apoplast redox level resulting in modification of receptors activities and signals transduction, hence regulating defense and growth in plants (Pignocchi et al., 2003).

MATERIALS AND METHODS

This research was conducted in experimental lab of Plant Biotechnology & Environmental Physiology Program, University of Florida. The homozygous salk lines for ascorbate oxidase were identified from *Arabidopsis* TAIR

search tool. Arabidopsis homozygous line (salk-108854) was obtained from *Arabidopsis* stock from ABRC (https://www.arabidopsis.org). The Arabidopsis ascorbate oxidase mutant and wild type columbia seed were germinated and grown under controlled conditions and seed were collected after flowering and seed setting (Figure 2).

The AO-mutant was considered and confirmed for T-DNA inserts. The DNA was extracted from both mutant and wild type plants by using CTAB method of nucleic acid purification (Doyle, 1990) and polymerase chain reaction (PCR) was performed.

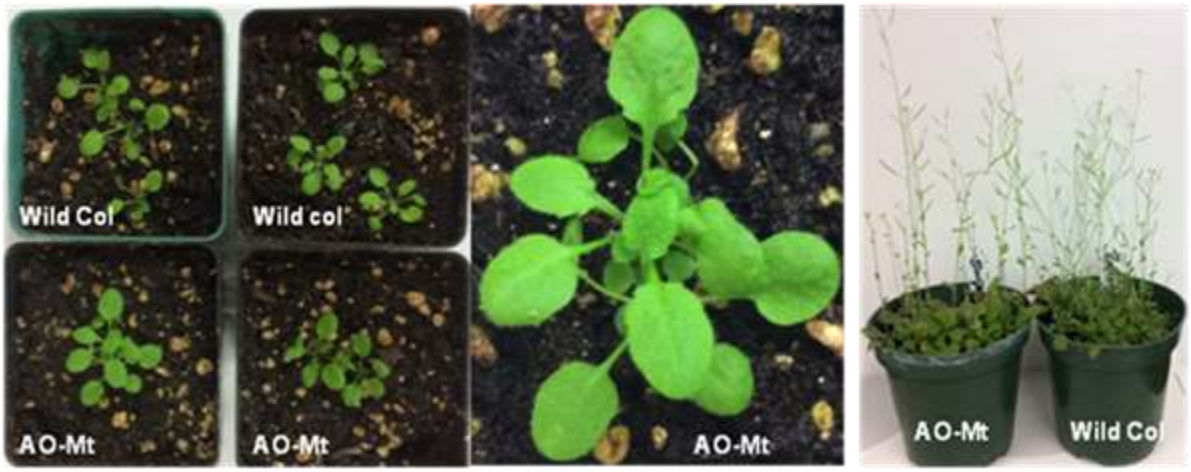

Figure 2. Whole *Arabidopsis* plant phenotype of wild type Columbia and AO-mutant from vegetative to flowering stage in pots.

For isolation of high-quality total *RNA* from *Arabidopsis* plant tissues, RNeasy Kit QIAGEN was used. The primer were designing from primer pick program in NCBI database based on T-DNA border as follow AO-F 5′ATGGGCTGATGGAGCAGC 3′ and AO-R 5′CCTCTTCCATTGATCAACAAGC 3′. Actin 2 was used as internal control gene withe the following primers sequence as follow ACTIN-2F 5′TTGGCATGTCATCACGATTGGT3′ and ACTIN-2R 5′TGCTTGGTGCAAGTGCTGTGAT 3′ in PCR analysis. For expression studies RNA was quantified spectrophotometrically and semi quantative qRT-PCR was performed for both AO mutant and wild type *Arabidopsis*

RESULTS AND DISCUSSION

The primary step for describing and validating the function of a gene is through the identification of knockout-mutant. Once the mutant line is isolated plant homozygous for the mutations need to be recognized, out-crossed, and examined to confirm the presence of a single T-DNA insertion. After confirmation of mutant under control the next step is to investigate the consequence of mutations on growth and developmental phases comparative to the wild types. The PCR screening methods for distinct knockouts mutation as a marker to study various physiological functions is very effectual and productive approach.

A large number of library of available T-DNA insertional mutant-lines are precious resource in area of functional genomics study for locating useful knockout alleles of each gene. In plants characterization and explanation of genes function depends mostly on study of T-DNA insertions-lines. Whether the insertions were created in coding or non-coding region of gene, it has similar effect of knocking-out the exon or an intron in the protein-coding region and is similarly effective in knocking out the targeted genes (86%). However it has little effective in case of insertion made before start-codon and after the stop-codon (Wang, 2008).

The efficiency of T-DNA insertions can be assessed generally by expression studies of genes mutation in the case of homozygous-insertional mutant. As detailed studies have also predicted that it could be detail below, this can be complex as the transcripts level possibly not be in correlation with protein levels (Delatte et al., 2005; Pastuglia et al., 2006) that was determined by the position of insertions in genes. In certain cases even not much significant difference was observed in level of transcript from that of wild one, as mutant not showing protein production (Monte et al., 2003), obfuscating the assessment

of T-DNA insertions specifically by use of reverse-transcription (RT-PCR) polymerase chain reaction.

During this study ascorbate oxidase mutants were studied by PCR. Gene specific primers based on T-DNA border were used to confirm the T-DNA insertional mutagenesis. RNA was also isolated and semi quantative RT-PCR was used to check the expression of AO-insert using ACTIN-2 as internal control. For this purpose both DNA and RNA were isolated from the mutant and wild type columbia plants. The RNA was used for one step RT-PCR analysis to see the expression of this gene of AO-mutant and wild columbia *Arabidopsis* insertion line. This particular PCR with genomic DNAs as template lead to only AO-Wt amplification of PCR products (Figure 3a and b) while AO-Mt plants do not contain T-DNA sequences confirming T-DNA insertion in knock out *Arabidopsis* mutants. This is the good indicator of whether a gene function is disrupted or absent in the mutant plant. Semi quantitative PCR showed AO transcripts of 309 bp in case of both wild type and mutant *Arabidopsis* plants as checked along with 100bp ladder. However altered phenotype was also observed in case of AO-mutants in case of low germination percentage, week flowers, week pods and reduced seed production etc. However the primers for standard PCR condition are often unsuccessful to provide the basic sensitivity for detecting infrequent T-DNA inserts in a population of collective T-DNA insertional lines.

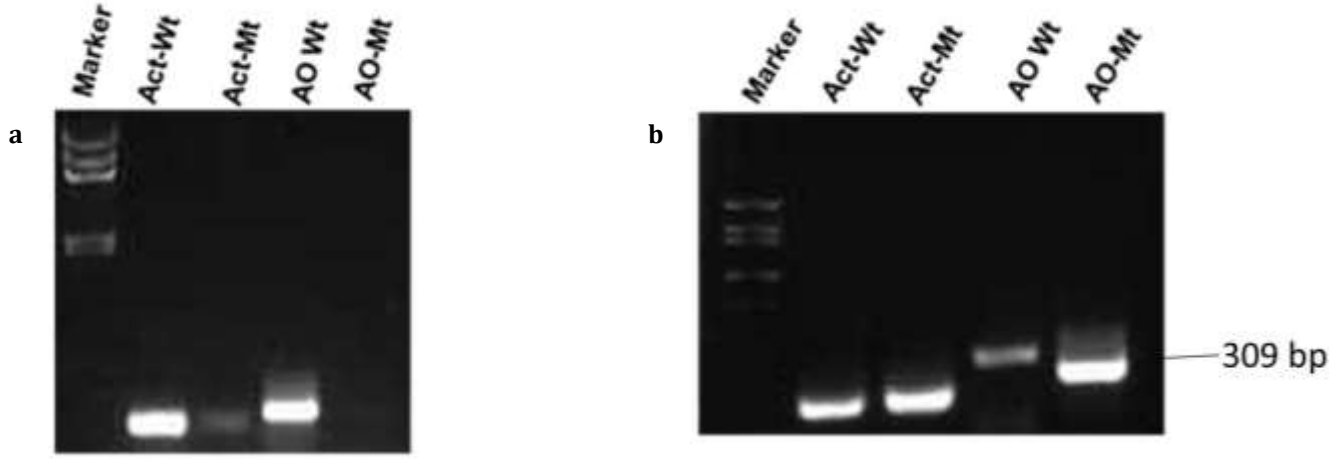

Figure 3. Detection of T-DNA insertion line by PCR and semi-quantitative Rt-PCR. (a) PCR on genomic DNA tests for the ability to amplify a genome region present in wild type and mutant line. (b). Semi-qRT-PCR of mutant and wild type AO transcript checked along with 100bp DNA ladder.

Once T-DNA lines have been selected, the next step was the isolation of homozygous segregates from parental plants that will be homozygous for this T-DNA insert. Thus PCR genotyping assay provided a potent and accessible method for identifying plant homozygous for this AO T-DNA insert successfully amplified a genomic region present in case of wild type plants or heterozygous lines but did not amplify gene in case of homozygous lines. Our results were according to the finding of Ronan *et al* who detected a band in wild type or heterozygous lines without any band in homozygous lines which may be due to the large size of T-DNA insert preventing amplification in PCR test, a dominant indicator for insert detection (O'Malley et al., 2015).

Previous studies have shown genotyping of T-DN Insertion lines using semi quantative RT-PCR reaction. This method was used to check the expression pattern of AO-inserts and in mutant and wild-type to ascertain the he capability of amplifying a region of genome that was present in both types of *Arabidopsis*. Efficacious genotyping depend on choice of good primer flanking the inserts position and mark the left-border of T-DNA (O'Malley et al., 2015). In some cases absence of transcripts of T-DNA disturbed genes was due to pre-mature termination of transcription after the failure of RNA polymerases to transcribe the complete gene (Matsumoto, 1994). Hence, this the clear indicator of absence of product of wild-type-PCR reaction as this line is homozygous for insert which will be further used to study the physiological role of AO under stress conditions.

CONCLUSION

The position of the T-DNA insertions in relation to gene-annotation is one of the prime indicators to analyze disruption or absence of gene functions in mutant. In this

study pooled T-DNA samples were screened that revealed the choice of salk-line primers of PCR for the T-DNA border region is critical to the optimizations of the procedure. Therefore we tested the primer set under the same PCR conditions for identifying T-DNA insertions to ensure compatibility. This study will help us to understand the role of AO enzyme in plants in plant defense in Reactive oxygen species using T-DNA mutagenesis.

ACKNOWLEDGEMENTS

This work has been developed and supported by University of Poonch Rawalakot Azad kashmir and in the framework of Plant Biotechnology & Environmental Physiology Program University of Florida USA.

CONFLICTS OF INTEREST

The authors declared no conflict of interest. The funders had no part in the design, collection analyses and interpretation and writing of short communication.

AUTHOR'S CONTRIBUTION

All authors contributed and supported towards writing of this short communication.

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