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Transgenic cassava lines carrying heterologous alternative oxidase (AtAOX1a) showed impaired quantitative and qualitative response to embryogenesis

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In our approach to control reactive oxygen species produced as a result of oxidative stress experienced by cassava roots during harvesting, a phenomena which causes postharvest physiological deterioration in the roots, we transformed cassava variety TMS 60444 with the *AtAOX1a* gene, driven by the 35S promoter, using agrobacterium-mediated approach. Extracted genomic DNAs of putative transgenic lines were screened using polymerase chain reaction technique (PCR). Messenger RNA was extracted from selected PCR-positive lines for reverse transcription-PCR analysis for gene expression. To screen positive lines for gene function, leaf lobes from two transgenic lines with a line carrying an empty vector and the wild type were subjected to somatic embryogenesis (SE), a known oxidative stress process. The results show that the wild type, at 16 days after initiation (DAI) of the leaf lobes on callus initiation medium, had the highest (100%) number of leaf lobes that produced at least one observed organised embryogenic structure (OES). This was followed by PEV-3, the empty vector plant with 50% OES production, while PB-3 had the least percent (20%) of leaf lobes with OES. PB-3 line also had no OES at all in five out of the seven periods of data collection. During the period, the wild type recorded the highest attainable OES quality score of 2.0 (on a scale of 1-5 where 1=bad and 5=excellent) at the first initiation cycle. Both the transgenic lines and the empty-vector plantlet recorded quality score of 1.0. It seems AtAOX1a only hinders OES development, but exerts little effect on the quality, if OES does not degenerate after development.

Key words: Genomic DNAs, reverse transcription-PCR, somatic embryogenesis (SE).

INTRODUCTION

Cassava is an important food crop for over 250 million people in the world (Sayre et al., 2011), and plays a major role in the food chain of nearly 600 million people across the world. There is great diversity in cassava germplasm for important traits, which has helped in the development of many cassava varieties that combined good agronomic qualities over the years. Conventional breeding has been successful in the improvement of traits such as yield, disease and pest resistance, and high starch and dry matter contents. However, the agronomic improvement of cassava (*Manihot esculenta* Cranz) using conventional breeding approaches has been hampered by a variety of factors including, low production of flowers, apomixis (Nassar et al., 2000;

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Abbreviations: ROS, Reactive oxygen species; **OES,** organised embryogenic structure; **PCR,** polymerase chain reaction; **BAP,** benzylaminopurine; **DAI,** days after initiation; **SE,** somatic embryogenesis.

Nassar, 1995), a long reproduction cycle, limited seed set (Hahn et al., 1990), variability in ploidy number, and inbreeding depression (Nassar, 2003). This has made the application of biotechnology in cassava crop improvement inevitable.

Biotechnology presents a tool that provides quick gene introgression into crop plants. Genes isolated from other sources have been successfully inserted into cassava genome (Taylor et al., 2001; Gonzalez de-Schopke et al., 1998; Uzoma et al., 2006; Siritunga and Sayre, 2003). The expression of various inserted genes into the cassava genome, which had resulted in increased disease resistance (Fauquet et al., 1992), modified starch quality (Salehuzzaman et al., 1993), reduced level of cyanogenic glucosides (Siritunga and Sayre, 2003), and extended shelf life of cassava roots (Thro et al., 1996), demonstrates the ability of genetic engineering to bring about rapid cassava crop improvement. The increasing focus in the use of cassava as an industrial raw material in the food, feed and energy industries (bioethanol) (Ceballos et al., 2007a) has necessitated the need to circumvent the major constraints to cassava production, of which biotechnology is an effective tool.

In order to increase the stress tolerance capacity of cassava through the prevention of over-accumulation of reactive oxygen species (ROS), cassava was engineered to over-express mitochondrial alternative oxidase by inserting alternative oxidase isolated from *Arabidopsis thaliana* into the genome of the cassava cultivar TIS 60444 through agrobacterium-mediated transformation. The gene was driven by the constitutive 35S promoter. Alternative oxidase is the terminal oxidase of a cyanide-insensitive pathway that helps to prevent the over-accumulation of ROS (H_2O_2) caused by such stress factors like wounding in cassava roots during harvesting, during transportation, and during the short period of heaping up before processing. The over-accumulation of ROS leads to postharvest physiological deterioration in cassava roots, which begins within 24 h after harvesting (Reilly et al., 2003, 2007), and renders the roots unpalatable and unmarketable within 72 h. The AtAOX1a is expected to reduce ROS accumulation in cassava roots, thereby increasing its shelf-life. In other to test the effectiveness of AtAOX1a in reducing the effect of over-accumulation of ROS (H_2O_2) in cassava, the putative transgenic lines carrying AtAOX1a were subjected to the oxidative stress system of de-differentiation process in somatic embryogenesis. Somatic embryogenesis is a stress-related procedure due to the activities of auxin which generates ROS (Pfeiffer and Höftberger, 2001) that plays a key role in signal transduction that leads to de-differentiation of the leaf lobes to organised embryogenic structure (OES). The transgenic lines carrying AtAOX1a were evaluated for the effect of the gene on the formation of OES from transgenic cassava leaf lobes. The over-expression of AtAOX1a is expected to prevent ROS accumulation, which is expected to negatively impact OES generation and quality. The preliminary results of the evaluation of the transgenic lines for rate of OES generation and quality are presented in this paper.

MATERIALS AND METHODS

Development of AOX1a transgenic lines

The AOX1a gene was sourced from Dr. Sayre's Laboratory at the Donald Danforth Plant Science Center, Saint Louis, Missouri, USA. The gene carried by the pBI121 binary vector and cloned in *Escherichia coli* was extracted from developed *E. coli* colonies using Qiagen DNA Extraction reagents and manual. The pBI121 DNA samples were screened for the insert using the polymerase chain reaction (PCR), and the PCR product run on agarose gel electrophoresis. The gene on pBI121 had been fused to patatin promoter. The patatin promoter was removed using the restriction enzyme XmaI, and 35S promoter was cloned into the position so that it can drive the expression of the gene in all parts of the plant. Thereafter, the complete gene cassette was cloned in *E. coli* and transferred to the binary vector pBI121. Positive pBI121 DNA samples were used to transform *Agrobacterium tumifaciens* strain LBA4404.

The Cassava cultivar TMS 60444 was genetically engineered by inserting AtAOX1a into it by co-culturing friable embryogenic callus of cassava TMS 60444 with *Agrobacterium* strain LBA4404 carrying the gene insert in the presence of acetosyringone (a compound that stimulates the transfer of the T-DNA of the agrobacterium into plant tissues) using the cassava transformation protocol developed by the International Laboratory for Tropical Agriculture Biotechnology (ILTAB), Donald Danforth Plant science Center. Putatively transgenic friable embryogenic callus were recovered on antibiotic medium and regenerated to mature cotyledon stage embryos. Whole plants were recovered on MS media (Murashige and Skoog, 1962) supplemented with benzylaminopurine (BAP) as stated by Sayre et al. (2011), and as used by ILTAB. Recovered *in vitro* plantlets were screened for the presence of the transgene using the polymerase chain reaction technique. To screen the putative transgenic lines for expression of the transgene at the transcript level in the roots, RNA was extracted from transgenic cassava roots, and cDNA was made from the RNA. The transgene expression was amplified using the cDNA as the DNA template through reverse transcription (RT)-PCR. To further demonstrate that the cDNA synthesized was of good quality, tubulin, a constitutive sequence in most plants was amplified from the cDNA to show the integrity of the synthesized cDNA. Positive events with good heterologous gene expression were micro-propagated and preserved at 28°C under light.

Organized embryogenic callus development:

In our experiment, somatic embryos were developed from leaf lobes collected from transgenic cassava lines carrying the AtAOX1a gene. Immature leaf lobes measuring about 1 to 6 mm obtained from about six weeks old *in vitro* derived plants were used. Excised immature leaf lobes were placed on solid DKW medium supplemented with sucrose (20 g/L) and picloram (50 uM), with the abaxial side and midrib of the explants pricked with needle and placed in contact with the medium for the induction of OES. De-differentiation of the leaf lobes to OES was expected to begin by 48 h after placement on DKW medium through the swelling of the leaf lobes.

Therefore, data collection was started at three days after initiation on medium, and continued at 5,7,9,11,13 and 16 days after initiation on the medium. At each period, number of leaf lobes, hence percent of leaf lobes with at least one OES was taken, as well as the quality of the OES.

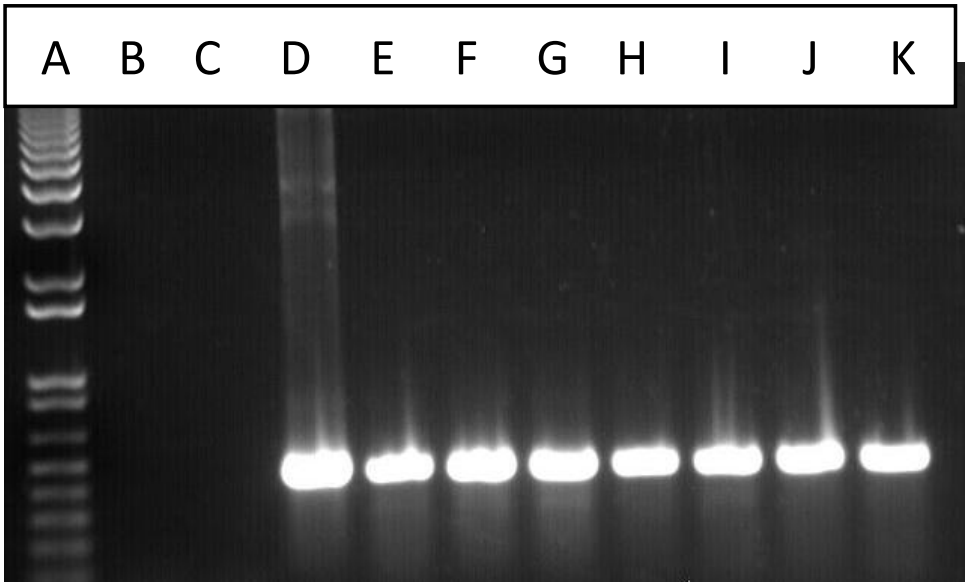

Figure 1. Band showing the presence of the AtAOX1a in PCR amplified product with extracted pBI121 DNA cloned in transformed *E. coli* during 35S:AtAOX1a:NosT cassette construction. A , Marker; B, wild type (WT); C, PCR mix without DNA (negative control); D, vector DNA (positive control), E to K represent positive transformed E. coli DNA samples.

OES quality scores and description of scores

1 = BAD: OES not very distinguishable from solid mass callus; OES attached strongly to solid callus and so need much trimming and cleaning.
2 = FAIR: Few OES found in mucilaginous callus; most OES still attached to solid callus. OES attached to callus will need much trimming and cleaning.
3 = GOOD: Few OES found in mucilaginous callus; most OES still attached to solid callus, but will need minimal trimming.
4 = VERY GOOD: Many OES found in mucilaginous callus; many OES still attached to solid callus but not strongly and so need little cleaning.
5 = EXCELLENT: Many OES distinct and found in mucilaginous callus, easy to pick with little or no cleaning.

RESULTS AND DISCUSSION

Gene construct is an important component of genetic engineering. Because the expression of every gene is regulated by a promoter, we fused the Cauliflower virus 35S promoter to the alternative oxidase AtAOX1a gene for constitutive expression. The bands from plasmic DNA cloned in and extracted from positive *E. coli*, colonies are presented in Figure 1. Base on the fact that the 35S was specifically fused to the gene, we also checked the presence of the 35S promoter in the gene cassette. The PCR amplification of the 35S promoter after insertion into the binary plasmid pBI121 is shown in Figure 2. The primers to amplify 35S for cloning into pBI121 binary vector carrying AOX1a:NosT cassette during the making of the 35S-AOX-NosT construct is given: Forward Primer: 35SFAscI1:5’ CAG GCG CGC CAG ATT AGC CTT TTC AAT TTC AG 3’- Tm=49.0; Reverse Primer: 35SREcoRI1: 5’ GCG AAT TCC GTG TTC TCT CCA AAT GAA ATG 3’- Tm= 52.1. Primers for screening the 35S-AOX fragment in transgenic events included: Forward Primer: 35S303F1 5’ GCC ATC GTT GAA GAT GCC TCT GC 3’Tm = 60.7; Reverse Primer: 35SAOX303R 5’ CATGGCCTGAAACAGTTCCACTTC 3’ Tm=58.4

Screening of transgenic lines

The routine generation of transgenic plants involves analysis of transgene integration into the host genome as well as the evaluation of the transgene expression using standard molecular techniques such as PCR, RT-PCR among others (Flachowsky et al., 2008). The presence of the transgene in selected putative transgenic lines is presented in Figure 3, and the agarose gel electrophoresis of RT-PCR to show the expression of the transgene (AtAOX1a) at the transcript level is presented in Figure 4. The tubulin bands that show the integrity of the synthesized cDNA from the PCR positive putative transgenic lines are also presented in Figure 5. Transcript expression of the genes is an indication that the transgene is being expressed in the target tissues of the plant. It is a procedure often used across crops to detect transcript signal, as used by Netrphan et al. (2012) in detecting mRNA expression in cassava.

Information on transcript analysis of the alternative oxides gene AOX1a in relation to embryogenesis in crops is scarce. However, in their work, Frederico et al. (2009)

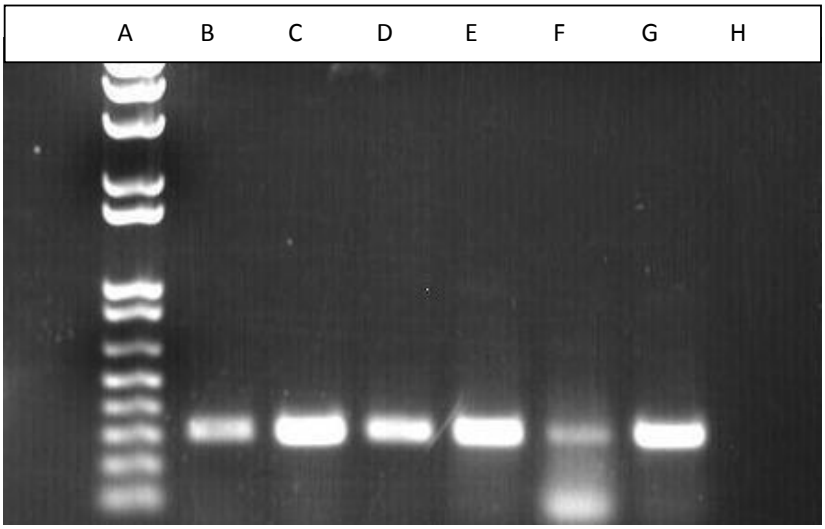

Figure 2. Agarose gel showing PCR amplification of 35S fragment using the primers above after insertion into the vector pBI121 DNA to make a complete 35S:AtAOX1a:NosT cassette. Lane A, Marker; B, vector DNA carrying 35S promoter; C, D, E, F and G are positive transformed *E. coli* DNA samples; H, negative control.

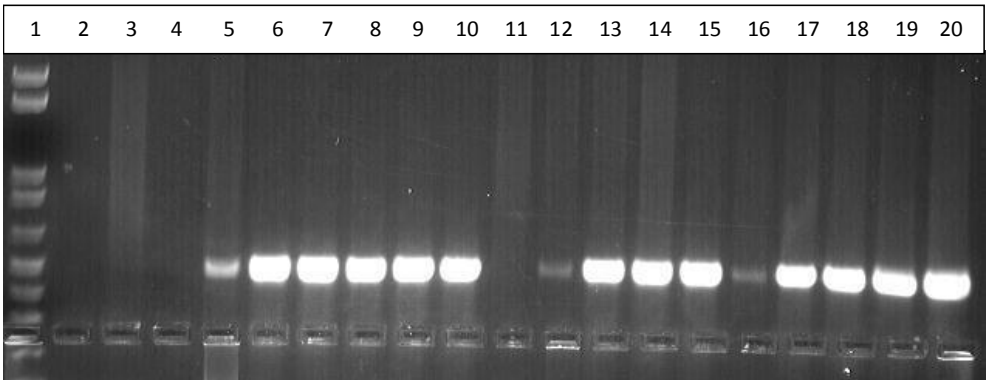

Figure 3. Some transgenic lines carrying the 35S-AOX fragment amplified using 35S303F1 and 35SAOX303R primers. Column 1, 1 kb-plus marker; columns 2, 3 and 4, negative controls (wild type), PCR mix without DNA, and plasmid DNA without transgene, respectively; 5 ,vector DNA with transgene (positive control); 6 - 20, represent putative transgenic events. Lanes without the band are false positives plantlets that do not carry the transgene.

reported a down-regulation of the DcAOX1a during somatic realization phase embryo and up-regulation of DcAOX2a. The down-regulation of AOX1a is due to the fact that the realization phase depends on auxin depletion. Somatic embryogenesis is an auxin-dependent process, so embryogenesis is a stress-inducing process. Therefore, the auxin-dependent phase is when AOX1a becomes important, and not the realization phase.

Response of putative transgenic lines to somatic embryogenesis

The results presented here show the response of the transgenic lines created through the processes discussed on somatic embryogenesis, which is a stress-related procedure. The over-expression of AtAOX1a is expected to prevent ROS over-accumulation that is a direct result of stress. ROS is essential for the de-differentiation process that leads to the development of OES. Thus, the over-expression of AtAOX1a is theorized to impact negatively on OES generation and quality. After the commencement of the experiment, only the lines carrying empty vector (negative control) (pEV-3) had initiated OES at 3 days after initiation (DAI) on DKW medium. At 3 DAI, 25% of the leaf lobes from pEV-3 had OES (Figure 6). By the 5th DAI, the two controls (wild type TMS 60444 and pEV-3) and the transgenic line pB-3 had developed OES.

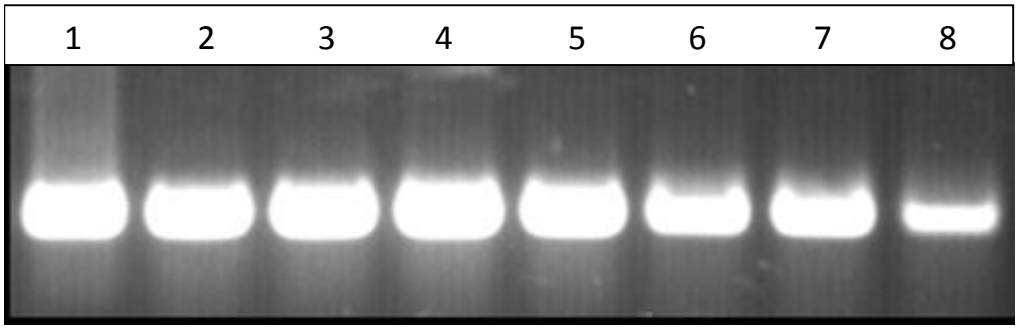

Figure 4. AtAOX1a transgene expression amplified from cDNA that was synthesized from the mRNA from the roots of selected transgenic lines. Lane 1, vector DNA (positive control), while Lanes 2 to 8 are cDNAs from PCR-positive transgenic lines to show transgene expression in the recovered plants.

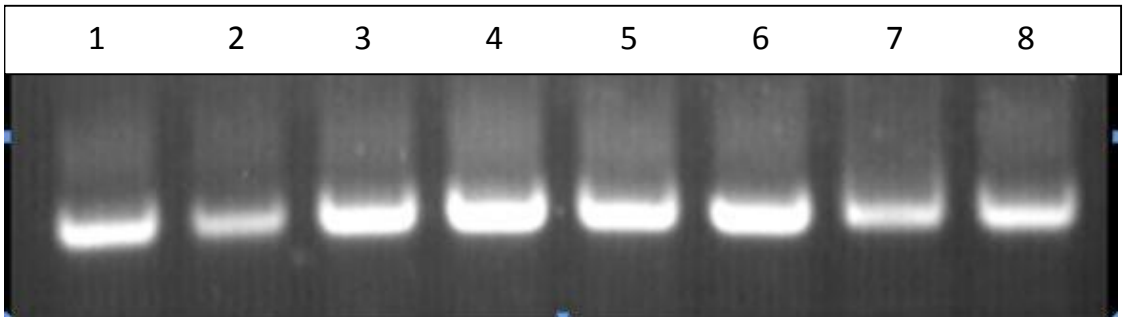

Figure 5. Tubulin expression in the cDNA of the transgenic lines. Lane 1, Plasmid DNA carrying the tubulin gene. Lanes 2 to 8 correspond to the cDNA used in Figure 4 above. The tubulin expression in the cDNAs implies that the integrity of mRNAs from which the cDNAs were synthesized, as well as the cDNAs was of good quality.

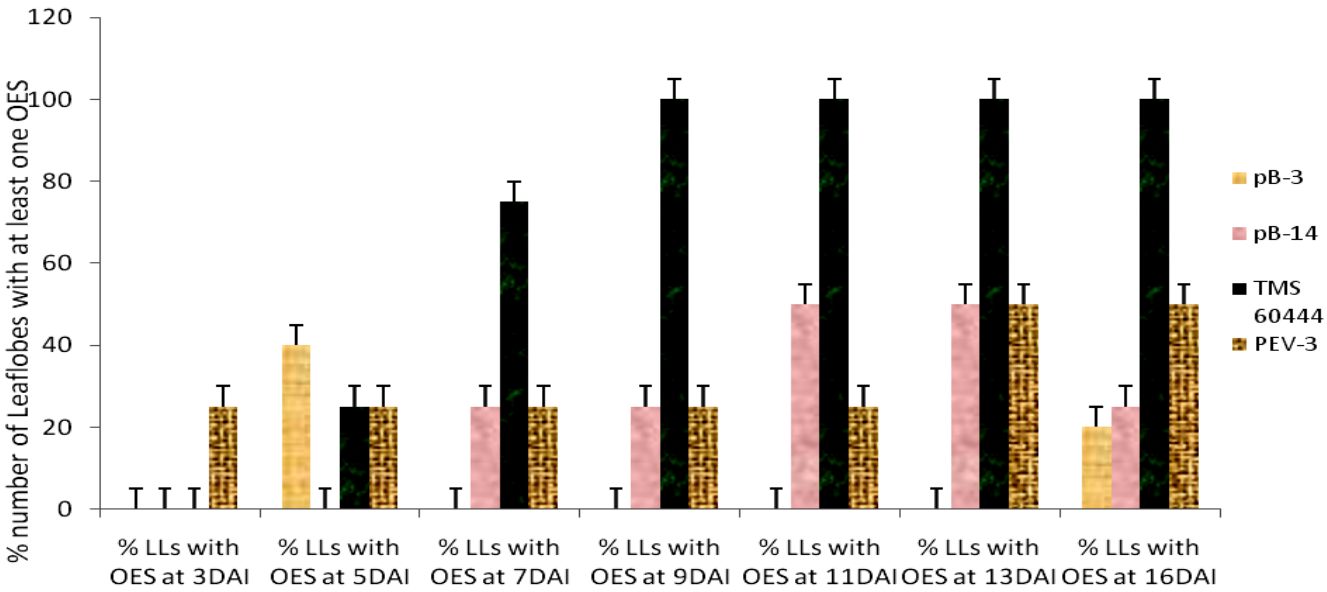

Figure 6. Response of AtAOX1a transgenic cassava lines to the development of organized embryogenic structures (OES) at 3, 5, 7, 9, 11 and 16 days after initiation on medium (DAI). *LL = leaf lobes.

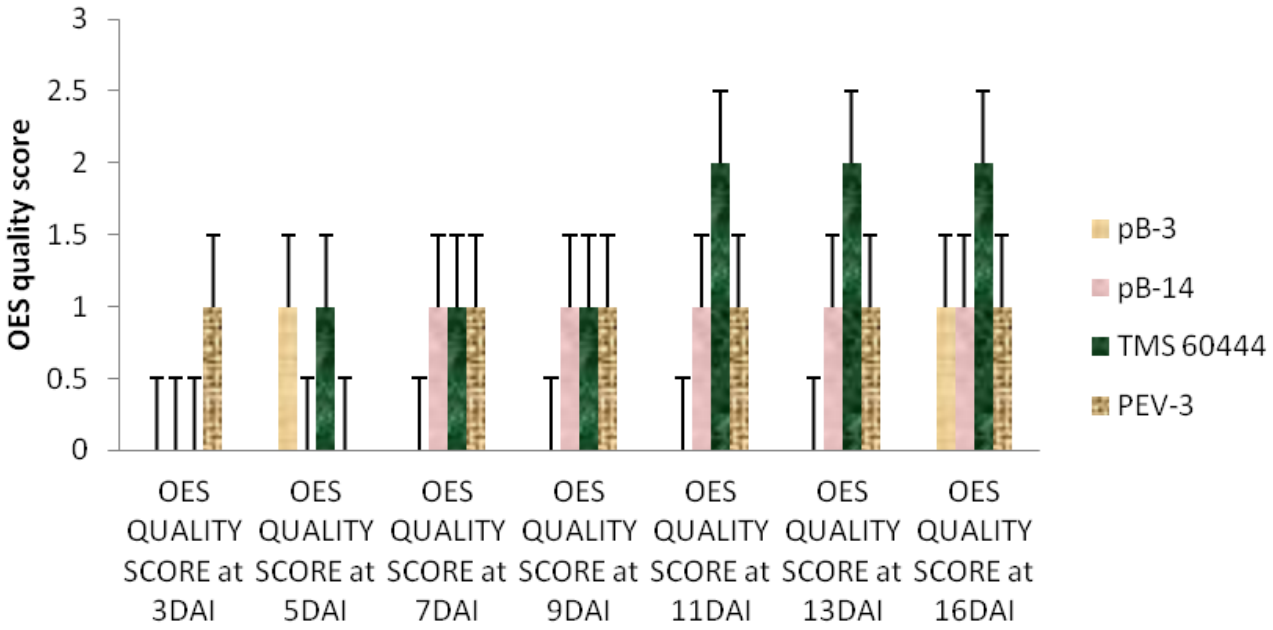

Figure 7. Quality scores of developing OES of AtAOX1a transgenic cassava lines and the controls at 3,5,7,9,11 and 16 DAI.

However, the wild type (TIS 60444) had almost 80% of the leaf lobes with OES, while both pB-14 (transgenic line) and the negative control (PEV-3) had 25% of the leaf lobes with OES by the 7 DAI. The OES of pB-3 had become undifferentiated callus, and so could not be counted anymore as OES. All the OES formed so far across the transgenic lines and the controls were of low quality with quality score of 1.0 (Figure 7). By 9 DAI, 100% of TMS 60444 (wild type) leaf lobes had developed OES, though the quality of the OES remained poor at 1.0. There was no difference between the performance of the transgenic lines at 7 and 9 DAI. Up till 16 DAI when the experiment was terminated, the 100% of leaf lobes from the wild type had developed and maintained OES, while the OES quality increased from 1.0 to 2.0 from 11 DAI till the end. As for the transgenic lines and the empty vector control (pEV-3), there were also changes. While 50% of the initiated leaf lobes of the transgenic line pB-14 and control pEV-3 had developed OES between 11 DAI and 16 DAI, pB-3 had maximum of 20% of the leaf lobes produced OES. The obvious and important difference between the OES produced by the transgenic lines and the non-transgenic lines is the fact that the OES from the transgenic lines were never maintained as they degenerate with time and fast too. The highest quality of the OES produced by the transgenic and the empty vector lines was 1.0.

Somatic embryogenesis (SE), according to Frederico et al. (2009), is the most prominent example of cell reprogramming, and has be interpreted as a stress-induced morphogenic response (Pasternak et al., 2002; Potters et al., 2007). The mitochondrion has been recognized to have strong effect on cell dedifferentiation (Amirsadeghi et al., 2007; Sheahan et al., 2005). The alternative respiration pathway is localized in the mitochondria, and Clifton et al. (2005, 2006) have pointed to the importance of this pathway as an early sensoring system for cell programming through the activities of the AOX. The enzyme is related to all types of abiotic and biotic stress and known to be involved in growth responses and development (Giraud et al., 2008; Ho et al., 2007). Therefore, the differential response to SE observed between the transgenic (lines especially pB-3) and the controls (TMS 60444 and pEV-3) was expected. This is because the presence of auxin in the DKW medium and leaf lobes excision and pricking all induced stress which usually leads to the over-production of ROS (H_20_2). AOX is a known scavenger of H_20_2, so the higher the expression of AOX in the mitochondria, the lower the H_20_2 accumulation, and the poorer the OES development anticipated since ROS is important in the cell de-differentiation process. Therefore, of all the lines, pB-3 had the worst OES development. No OES was produced for most part of the trial. As observed, pB-14, another transgenic line, on the average, had similar OES development and quality as pEV-3, the empty-vector control. However, it could be observed that while pEV-3 had 25% of its leaflobes producing OES as early as 3 DAI, pB-14 did not reach that level of OES development until 9 DAI. The similar level of production between pEV-3 and pB-14 in later periods could be due to any of such situations as low level of AOX production, or the insertion of the gene disrupted another important gene in the stress cascade moderation. Therefore, further analysis

needs to be done to determine the cause of the observed high OES development in pB-14.

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