REDUCING POST-HARVEST PHYSIOLOGICAL DETERIORATION IN CASSAVA BREEDING BY NATIONAL ROOT CROPS RESEARCH INSTITUTE UMUDIKE

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Abstract
Cassava roots are notable for their short shelf-life due to post-harvest physiological deterioration (PPD). PPD is initiated by mechanical damage, which typically occurs during harvesting and progresses from the proximal site of damage to the distal end, making the roots unpalatable within 72h. The short shelf-life severely limits marketing options by increasing losses, marketing costs, and limiting access to urban markets and processing centres. Reducing PPD of cassava roots is amongst top research priorities National Root Crops Research Institute (NRCRI) Umudike. Our main approaches NRCRI aimed at reducing Cassava PPD include conventional breeding, mutagenesis, molecular breeding and genetic engineering. Land races and exotic genotypes have been screened for delayed PPD. Limited variability was observed amongst land races in this trait. Thirty three genotype with delayed PPD at 7 days after harvesting (DAH) and 22 with delayed PPD at 14 DAH have been identified from backcross populations of Manihot walkerae. Gamma radiation was used to induce genetic variation for delayed PPD in local germplasm. Mutagenized populations were developed using in-vitro plants and OP seeds of farmer preferred varieties and land races. Few genotypes from these populations had low PPD (7DAH). Genetic mapping for PPD using genomic DNA isolated from young leaves of parental genotypes indicated that genetic factors, most likely major QTLs, were likely involved in the expression of delayed PPD in cassava. Another strategy adopted was to use the synergistic effect of over expressing nuclear encoded gene, alternative oxidase (AOX) and increased accumulation of beta carotene content in cassava roots to delay onset of PPD. Constructs carrying appropriate genes were successfully transferred into cassava and plants expressing the inserted genes have been obtained and are being evaluated appropriately. Preliminary success has been achieved in developing materials with delayed PPD. Further evaluation of these materials at different locations is necessary due to high influence of G x E interaction on the trait. Furthermore, markers for PPD are being developed, and image analysis method of assessing roots for PPD is being introduced to help straighten and fast-track the selection of genotypes with delayed PPD.

Keywords: Gamma radiation, molecular breeding, genomic breeding, genetic engineering and delayed PPD

Introduction
Cassava is a resilient crop with relatively high water-use efficiency compared with many crops. It is relatively tolerant of high soil acidity and low levels of soil phosphorus (P), which are both limiting factors in many tropical soils. It therefore does well in good soils and cope well when grown under marginal conditions. Due to its relatively high starch yield, cassava roots has myriads of uses: fresh market, processed foods, commercial starches and many starch-derived products, medicinal alcohol, feed for livestock, and more recently as a bioenergy feedstock (Kueneman *et al*., 2012). The roots can remain in the ground for several months without serious deterioration therefore allowing for piece-meal harvesting when needed which is suitable for small scale subsistent purposes. However, once harvested and exposed to air, cassava roots are notable for their short shelf-life due to rapid PPD. Cassava has the

shortest shelf-life of any tuber crop (Ghosh *et al*., 1988). Post-harvest physiological deterioration is a ubiquitous phenomenon well known to stakeholders in the Cassava value chain. Symptoms of PPD include blue/black vascular streaking, brownish occlusions, and chemical deposits from wound sites, discoloration of the storage tissues, unpleasant flavor and odor (Reilly *et al*., 2007).

In ancient times, it was believed to be caused by lightning which accompanied tropical thunderstorms. Another myth has it that it was caused by witches who came at night to sweep away the intransigence of humans. Presently, available reports suggest that PPD results from the failure of wound response in cassava roots (Wenham, 1995; Beeching *et al*., 1998). Reactive oxygen species accumulation, a product of wounding associated with cassava harvesting, bulking and haulage process is implicated for the rapid PPD of cassava roots. Scientific research on the processes and pathways leading to PPD seem to be converging to oxidative stresses that may be associated with alternative respiratory pathways and potentially cyanide production (Sayre *et al*., 2011; Xu *et al*., 2013; Vanderschuren *et al*., 2014). The oxidative burst of Reactive Oxygen Species (ROS) leads to the spontaneous over accumulation of H_2O_2 which in turn leads to the release of defense enzymes in cassava roots. The bye-product of enzymatic action in the root is the brown colouration of the root flesh of fresh cassava within 24-72 hours after harvesting (Afuape *et al*., 2010).

The process of post-harvest deterioration in cassava can be physiologically sub-divided into two distinct stages, early and late events. The early events (30 minutes to one day after harvest) include a rapid, cyanide-induced burst of reactive oxygen species (ROS) production which initiates a programmed cell death pathway. This is followed by a further wave of cyanogenesis from the proximal (cut) to the distal end of the root (Iyer *et al*., 2010). Late events (after 1-2 days) include microbial and fungal infections and root deterioration. The potential of cassava as a market and food security crop is limited by its short shelf life. This short shelf-life of cassava roots severely limits marketing options by increasing losses, marketing costs, and limiting access to urban markets and processing centres. Wenham, (1995) estimates losses due to PPD in cassava to range from 5-25%. In SW Nigeria, it is estimated that 40 percent of total cassava produce is lost due to spoilage. According to a recent ex ante estimate, extending the shelf life of cassava to several weeks would reduce financial losses by $2.9 billion in Nigeria alone over a 20-year period (Rudi *et al*., 2010).

Environmental factors affecting the rate of PPD include temperature, humidity and oxygen (Zidenga, 2011). Careful manipulation of these conditions can help in managing PPD. For example, storage at 10°C and 80% humidity, waxing and careful avoidance of physical damage can all delay PPD significantly (Wenham, 1995; Rickard, 1985; Plumbey and Rickard, 1991). Cultural management methods which may be useful in delaying the onset of PPD include: horizontal placement of stems at planting which enhances the production of uniform tubers aligned at angles which makes them easier to pullout with minimized injury during harvest; pruning plants at least 6 days before harvesting which have been shown to reduce PPD though trading off dry matter by converting starch to sugar (suitable for boil and eat situations) (Tanaka *et al*., 1984; Plumbley and Richard 1991); and harvesting when the soil is wet or agitating hard soil around roots and then gently pulling them out greatly minimizes root injury. Storage conditions and practices can also go a long way in controlling PPD (Booth, 1975; Ravi *et al*., 1996; Oirschot *et al*., 2000; Aristizabal and Sánchez, 2007).

Inspite of these, reducing PPD remain amongst most important challenges facing cassava farmers. Consequently the development of delayed PPD (longer shelf-life) varieties of cassava remains one of the most important goals of cassava breeding and biotechnology and remains a top research priority

undertaken by National Root Crops Research Institute Umudike. This paper reviews the efforts of NRCRI Umudike at developing cassava genotypes with delayed PPD

Breeding Approaches at Overcoming Cassava PPD at NRCRI Umudike

Conventional breeding

Land races and improved genotypes have been screened for delayed PPD. Limited genetic variability was observed amongst land races in this trait. Thirty three genotype with delayed PPD at 7DAH and 22 with delayed PPD at 14 DAH have been identified from backcross populations of *Manihot walkerae* (Ewa *et al*. 2011; 2012).

Mutagenesis

Gamma radiation was used to induce genetic variation for delayed PPD in local germplasm. Mutagenized populations were developed using in-vitro plants and OP seeds of farmer preferred varieties and land races. Invitro plant materials from TMS 95/0379 and TMS 98/0002 were sent to the IAEA at Vienna for irradiation. These materials were irradiated at various levels: 12GY and 15GY. The irradiated materials were then micro-propagated to reduce chimeras over three cycles and then planted on the field (Egesi *et al*., 2009; Okogbenin *et al*., 2011). For invitro plants, 59 genotypes showed less than 5% deterioration (Table 1). The genotypes were both at dosage levels for 12GY and 15GY, although results indicate that 15GY accounted for roughly 66% of the materials that showed delayed PPD. TMS95/0379 had the highest number of genotypes with delayed PPD at 14 DAH. Nine M_1 genotypes were identified as having delayed PPD after 2 years of evaluation.

Table 1: M1 populations developed from irradiated in vitro plants showing delayed PPD

Parents	Irradiation level	Number of genotypes with low PPD (< 5%)	% genotypes with low PPD
TMS 95/0379	12GY	3	5.058
TMS95/0379	15GY	33	55.93
TMS98/0002	12GY	11	11.64
TMS98/0002	15GY	12	20.34
Total		59	100

Similarly, 2000 open pollinated seeds (M0) from 4 varieties AR 15-5, AR9-62, TMS30555, and TMS30572 were irradiated. Dosage applied includes 150GY, 200GY, 250GY and 300GY. Irradiated seeds (M1) were germinated and planted as a nursery. At least one commercial root type was harvested per genotype. The use of single plant imposed limitation to sample size obtainable which was a further constraint to elaborate quantification of PPD. At 12 MAP, rots were harvested and sectioned using the protocol as described by Wheatly (1985) (Fig 1). Two evaluations were made on the 7^{th} and 14^{th} day after harvest (DAH). Among seedlings evaluated, 9 genotypes had less than 5% deterioration. The 9 genotypes were all obtained from the irradiation dosage of 200GY and most were at 7DAH (Table 2)(Egesi *et al*., 2009).

Table 2: M1 populations developed from irradiated seeds showing delayed PPD at 7 and 14 DAH

Family	Radiation dosage level	Number of seedling generated with low PPD (7DAH)	Number of seedlings generated with low PPD (14DAH)
AR 15-5	200GY	4	0
AR9-62	200GY	2	0
TMS 30555	200GY	1	1
TMS30572	150GY	0	0
TMS30572	200GY	1	0
TMS30572	250GY	0	0
TMS30572	300GY	0	0
Total		8	1

The selected genotypes are undergoing further testing for PPD considering that the trait is highly influenced by G x E (Cortes *et al*., 2001). M1 genotypes not showing delayed PPD were self-pollinated to eliminate chimeras typically found in this generation to allow all recessive traits to express. The self-pollinated seeds will constitute the M2 generation. Result suggests that dosage level 15GY for invitro plantlets and 200GY for seeds gave the best response to PPD. Irradiation could lead to the silencing of genes for the expression of PPD thereby allowing their discovery in the M1.

Fig 1: Scoring for PPD

Molecular Breeding

A mapping population consisting of a half-sib backcross population (BC_1) was developed using CW 429-1 as donor parent to two elite but susceptible genotypes (MTAI 8 and SM 909-25). The BC1 population 122 individuals (66 from MTAI 8 and 56 from SM 909-25) was established invitro from embryo axes, micro-propagated and 4-10 plants per genotype were established in the field. Genetic mapping for PPD was carried out using genomic DNA isolated from young leaves of parental genotypes.

Ten markers were significantly associated with putative QTLs (Table 3) with phenotypic variance explained (PVE) ranging from 10 to 30%. The markers were distributed in different linkage groups suggesting that different genes were involved. The discovery of QTLs with large PVE indicated that major QTLs were likely involved in the expression of delayed PPD in cassava. PPD has been reported to be putatively involved in self-defense mechanism and several genes in this pathway have been identified (Reily *et al*., 2003, Cortes *et al*., 2002). It was observed that all the markers with putative QTLs associated with delayed PPD were derived from the donor parent CW 429-1 with positive additivity, indicating that delayed PPD was due to the presence of alleles contributed by CW 429-1 and thus the existence of genetic factors responsible for the trait.

Table 3: Markers significantly associated with QTLs for delayed PPD

Family	Marker	F	RSq	P
BIPD284	EST93	16.82	0.3013	0
BIPD284	EST209	16.02	0.286	0
BIPD284	rNS300	7.17	0.1504	0.0014
BIPD284	EST271	4.74	0.1059	0.0113
BIPD284	SSRY330	4.63	0.1026	0.0125
BIPD289	EST60	6.42	0.1893	0.0031
BIPD289	EST98	4.23	0.1333	0.0196
BIPD289	rSSRY330	3.57	0.1149	0.0349
BIPD289	SSRY150	3.55	0.1143	0.0355
BIPD289	SSRY67	3.29	0.1069	0.0447

Genomic breeding

National Root Crops Research Institute Umudike in collaboration with Dr Ale Gore of Cornel University are developing high pro-vitamin A, PPD tolerant cassava lines adapted to Nigeria via genomic selection to reduce root losses and improve the nutritional and economic value of this staple crop. This research is supported by Bill and Melinda Gate Foundation PEARL Award won by Dr Damian Njoku.

Genetic engineering

Another strategy employed in the attempt at controlling postharvest physiological deterioration (PPD) in cassava was genetic engineering. Genetic engineering (also known as genetic modification) is the process by which the genome of an organism is manually changed with the aim of making the organism acquire new traits it does not previously have, or to enhance the expression of a trait that was not well-expressed before. General model for genetic engineering procedure in cassava is shown in Figure 2. Some of the studies carried out by the NRCRI in partnerships with other global institutions focused on strategies that target the control of the onset of oxidative stress which has been implicated in the initiation of PPD (Reilly et al., 2003).

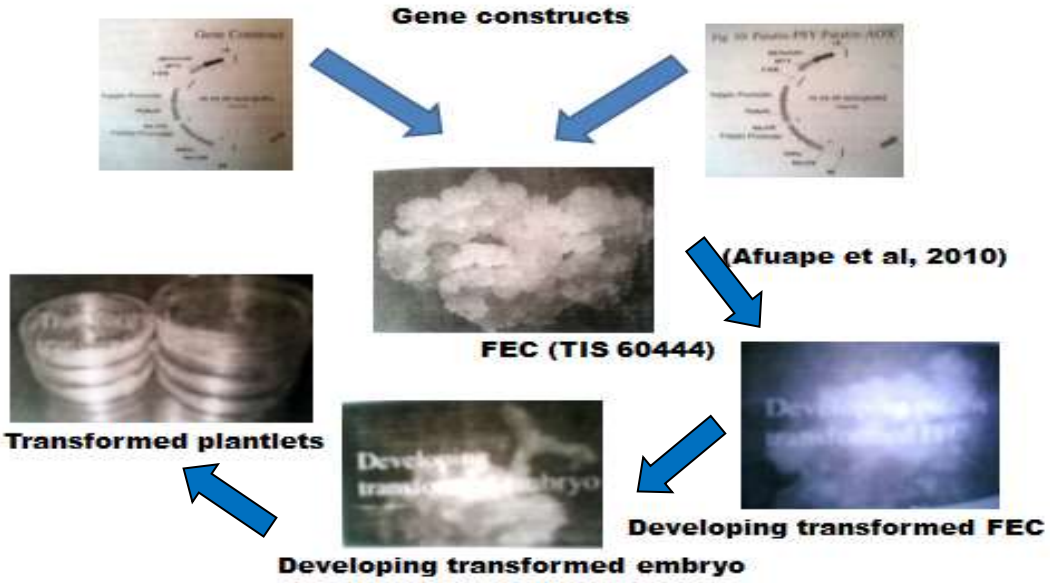

Fig 2: Transformation of cassava with gene constructs to reduce PPD

The carotenoid accumulation approach

Carotenoids are powerful anti-oxidants which, among other functions, mitigate the generation of reactive oxygen species (ROS) (Niyogi, 1999). The over-accumulation of ROS signals the onset of PPD. Increased carotenoid oxidation by ROS as a result of over-accumulation of ROS therefore plays a critical role in the sensing and signaling of oxidative stress condition (Havaux, 2014), thereby enabling the plant to deploy its defense mechanism in the process which leads to the prevention or delay of PPD. The PSY plays a key role in the process of the synthesis of carotenoids (carotenogenesis) (Rodriguez-Concepcion et al., 2001). It is believed that cassava has three PSY genes exist naturally in cassava ((Arango et al., 2010; Carvalho et al., 2016). The DXS (1-Deoxy-D-Xylulose-5-Phosphate Synthase) is also an important catalyst in the carotenoid flux regulation (Nisar et al., 2015). The over-expression of DXS has been reported to cause increased carotenoid production (Estevez et al., 2001; Carretero-Paulet

et al., 2006) due to its ability to induce and enhance PSY expression (Rodriguez-Concepcion et al.. 2001).

Through the Bio-Cassava Plus Project that aims at bio-fortifying cassava with pro-vitamin A, transgenic cassava lines over-expressing carotenoid content (up to 40% increase over the wild type) were developed by the insertion of the DXS-PSY gene cassette at the Donald Danforth Plant Science Centre. Fregene et al. (2010), using the carotenoid accumulation strategy, reported that over-expression of carotenoid content in the transgenic cassava lines led to delayed PPD of up to 28 days in the lines over-expressing carotenoid content (Figure 3).

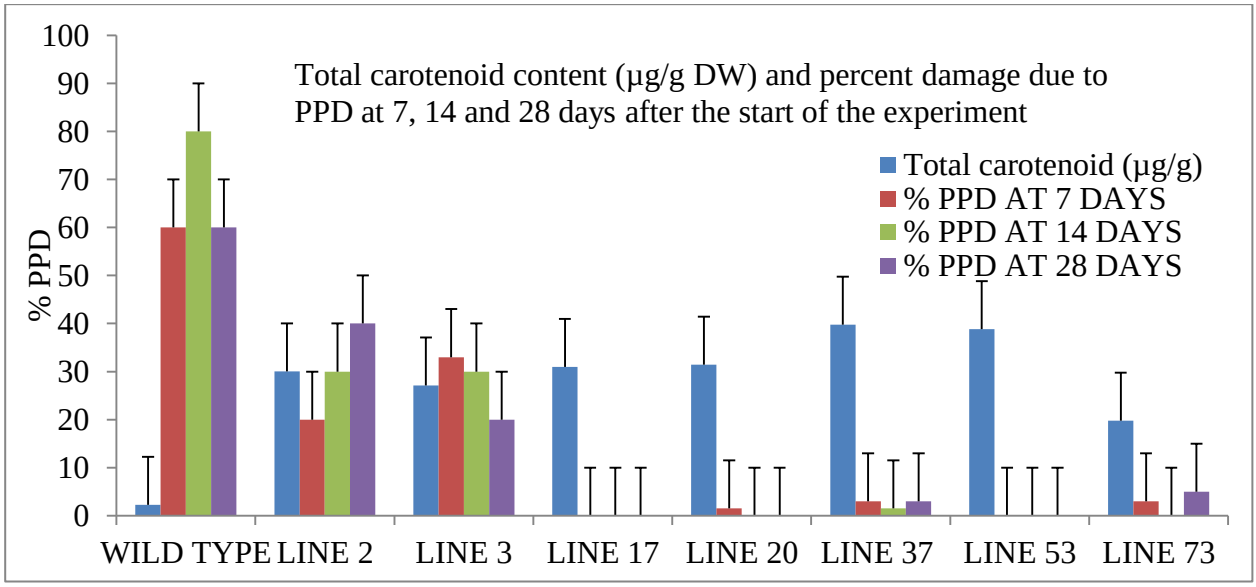

Figure 3: Total carotenoid (µg/g), and percent PPD at 7, 14 and 28 days after storage (Fregene et al., 2010).

Phytoene snthase (PSY) - Alternative oxidase (AOX1a) strategy

Another strategy employed in the control of oxidative stress to delay onset of PPD was to employ the synergistic effect of over expressing nuclear encoded gene, alternative oxidase (AOX) and phytoene synthase (PSY) to enhance the mitochondria alternative oxidase pathway to detoxify the excess ROS production during oxidative stress and to increase the beta-carotene content in cassava roots to delay onset of PPD, respectively. A small percentage of the inhaled oxygen ends up in the formation of ROS. As tissue respiration increases as a result of the stress imposed on the roots during harvesting, more ROS than usual is produced due to the ability of cyanogenic glucoside (cyanide) in cassava roots to incapacitate the Cytochrome c pathway. The alternative oxidase (AOX1a) pathway, which is present in most plants, therefore provides a backup pathway through which ROS accumulation is prevented when Cytochrome c is incapacitated. The prevention of excess accumulation of ROS prevents signal transduction in the root tissues, leading to the non-release of the defense mechanisms of the plant which causes PPD in cassava roots. Hence, the strategy was to enhance the accumulation of beta-carotene content and to over-express the function of alternative oxidase in the roots to detoxify ROS produced during oxidative stress.

The two gene constructs used:

1. PP-PSY-PP-AOX/pKAN2: Phytoene Synthase (PSY, which plays a key role in the biosynthesis of carotenoids and codes for beta-carotene accumulation) and the Alternative Oxidase (AOX, which serves as an alternative electron channel in the mitochondrion, thereby preventing

excessive reactive oxygen species (ROS) accumulation) were fused together, using a root expressing Patatin promoter and Nos terminator

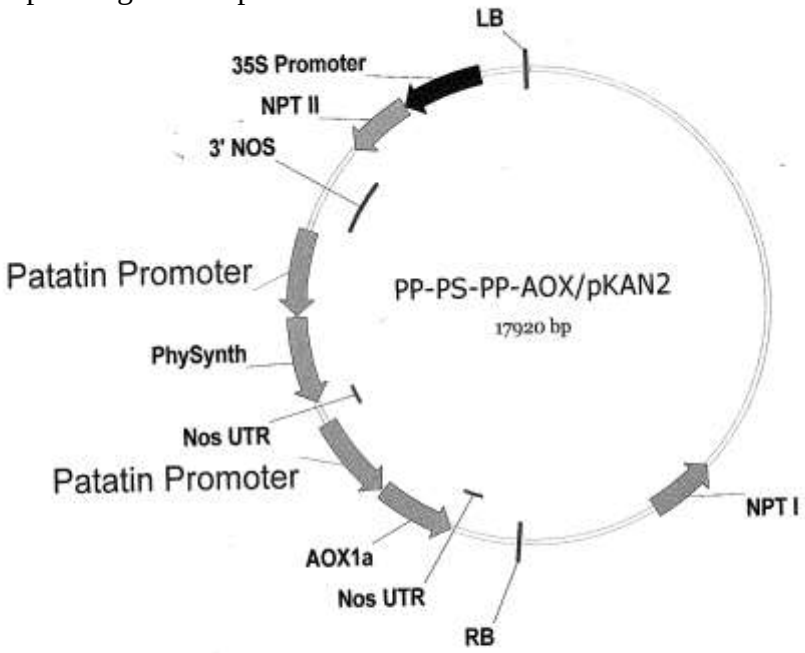

Figure 4: Patatin-PSY:Patatin-AOX gene cassette

2. P35S-AOX1a/pB1121: a single construct of Alternative Oxidase (AOX1a) gene was fused with a constitutive promoter (35S) in a plasmid pB1121 background for expression in all plant tissues.

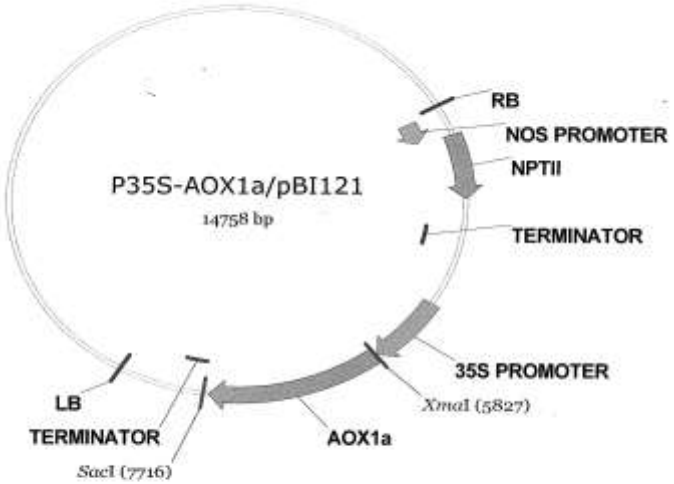

Figure 5: p35S-AOX1a gene construct

The two constructs carrying these genes were used to transform *Agrobacterium tumefaciens* strain LBA4404 which was then co-cultured with friable embryogenic callus (FEC) generated from leave lobes of Cassava cultivar TIS 60444. Through this procedure, these constructs were successfully transferred into cassava and putative lines expressing the inserted genes were selected (Fig 6). Details of the work on the single construct (P35S-AOX1a/pB1121) are presented in Afuape et al. (2013).

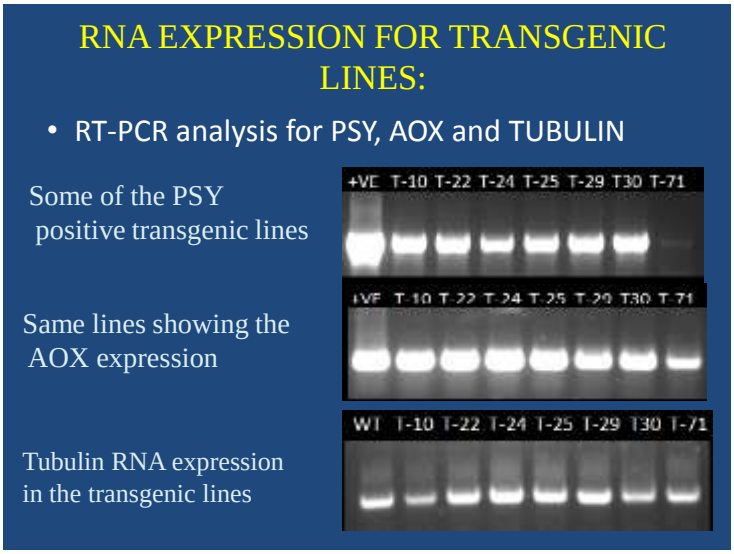

Figure 6: Some of the transgenic lines expressing inserted AOX and PSY genes at the transcript level

Conclusion

Preliminary success has been achieved in developing materials with delayed PPD. Evaluation of these materials at different locations is necessary due to high influence of G x E interaction on the trait. Furthermore, markers for PPD are being developed, and image analysis method of assessing roots for PPD is being introduced to help straighten and fast-track the selection of genotypes with delayed PPD.

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