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Characterisation of the Barley Oxalate Oxidase Gene and Generation of Rice Transformant

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Abstract: In the last two decades efforts have been made to identify, isolate and characterise disease resistance or defense related genes. One such gene is the oxalate oxidase (OXO; EC 1.2.3.4) gene which is an enzyme that enhances plants disease resistance by catalyzing the conversion of oxalic acid to hydrogen peroxide. Here, we isolated and transformed a putative *OXO* gene from a barley BAC clone 450N4 through PCR using specifically designed primers based on the barley OXO cDNA sequence from NCBI. The amplified sequence was identical to the barley *OXO* gene (Y14203) and barley OXO germin protein. The amplified gene has a coding region of 675 bp and has been shown to be involved in abiotic and biotic stress regulation. The OXO sequence was then introduced into a binary vector that was built using the pGA1611 and pCambia1301. This gene was then transformed into *Oryza sativa* indica cultivar MR81. The resulting construct, pCam-Ubi-MyOXO has a maize ubiquitin promoter and a NOS terminator. This construct was transformed into induced calli from rice immature embryo of a local elite cultivar, MR81, as explant. PCR analysis detected the presence of *MyOXO* gene with transformation efficiency of 1.7% (5/280). As a defense related gene and the role it plays in biotic stress regulation makes OXO a food candidate for resistance against diseases rice.

Key words: Oxalate oxidase, disease resistance gene, rice transformation, stress regulation

INTRODUCTION

Rice is a staple food of the 1/3 of the world population (Zhou *et al*., 1998). Supplying the demand of the increasing world population especially in Asia where it is largely dependent on rice as a food source is impeded by various factors such as the lack of agricultural land, availability of proper irrigation, climatic changes and biotic as well as a range of abiotic stresses. Diseases caused by bacteria, fungi and viruses have been known to cause large scale losses in rice farming regions. In Malaysia, the rice blast disease which is caused by the fungal pathogen *Magnaporthe oryzae* causes large scale losses to farmers and in certain instances 100% yield loss. On the overall diseases in rice cause a loss in income of about US$ 55 million each year (Kuyek, 2000).

Oxalic Acid (OA) is a key pathogenicity factor that is secreted by *Magnaporthe oryzae* to overcome the host (Dong *et al*., 2008). In resistant host, the production of Oxalate Oxidase (OXO), an enzyme that belongs to the germin family of proteins, is known to catalyses the degradation of OA to carbon dioxide and hydrogen peroxide (H_2O_2). By converting OA, it reduces tissue damage caused by acidification, sequestration of calcium that may weaken plant cell walls and also prevents OA from inhibiting the phenolic compounds in plants that functions in plant defense (Kuyek, 2000). Meanwhile, H_2O_2, a byproduct of the degradation OA has a crucial role in plant defense system (Hu *et al*., 2003). A few micro molar concentrations of H_2O_2 may reach levels that are directly toxic to microbes (Hammond-Kosack and Parker, 2003) and further contribute towards structural reinforcement of plant cell walls and trigger lipid peroxide and SA synthesis. Lipid peroxides and SA are known to have roles in signal transduction cascades that coordinate various defence responses (Morris and Bryce, 2000). Given the above reason it is expected that the over-expression of *OXO* within the plant system may result in elevated defence response in the host.

The *OXO* gene is long known as a marker of growth on set in germinating cereals (Thompson and Lane, 1980; Lane, 1994) and has been implicated as an agent in host-plant resistance to disease (Lane, 1994) through the central role of regulating the hypersensitive response (Lane, 1994). The barley *OXO* gene has been used in the breakdown of OA to H_2O_2 in transgenic soybean (*Glycine max*) (Donaldson *et al*., 2001) and sunflower (Hu *et al*., 2003). It had shown promise in controlling *Septoria musiva* in *Populus euramericana* (Liang *et al*.,

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2001), *Sclerotinia minor* in peanut (*Arachis hypogaea*) (Livingstone *et al*., 2005) and *Cryphonectria parasitica* in *Castanea dentata* (Marsh.) Bokh (Welcha *et al*., 2007). In addition to these reports, oilseed rape transformed with the *OXO* gene demonstrated enhanced resistance to OA generating fungi (Dong *et al*., 2008).

The purpose of this study was to isolate, characterise and validate the ability of a defence related gene, *OXO* in affording broad spectrum disease resistance to diseases in rice. In this study, we demonstrated the results of the isolation and *in silico* characterisation of the *OXO* gene from barley BAC clone. This gene was then used in the construction of a transformation cassette which was efficiently transformed into *Oryza sativa indica* var MR 81 via *Agrobacterium*-mediated transformation. These transgenic lines are currently being evaluated for their efficiency against various isolates or *Magnaporthe oryzae* obtained from the field in Malaysia.

MATERIALS AND METHODS

Construction of plant transformation vector: This research was conducted at the School of Bioscience and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Plant growth and screening was conducted within the greenhouse facilities adhering to international standards. The plant transformation vector was constructed using the *OXO* gene from barley BAC clone from the Clemson University Genomic Institute (CUGI), USA together with two binary vectors pCambia1301 and pGA1611 that was provided by Dr. K. Harikrishna from Sime Darby Technology Centre Sdn. Bhd., Malaysia and Dr. GynAng, of the Plant Functional Genomics Laboratory, POSTECH Biotech Centre, Korea. The BAC DNA from the barley clone 450N4 that contains the *OXO* gene was extracted using the alkaline lysis extraction method (Sambrook *et al*., 1989). The DNA was digested with *Not*1 and fractionated via Pulse Field Gel Electrophoresis System at 6V/cm at 14°C for 15 h (Initial time is 5 sec while the final time is 15 sec). The size of BAC DNA was determined as 106 kb. The barley *OXO* gene was amplified by Polymerase Chain Reaction (PCR) using the primers that were designed based on the barley OXO cDNA sequence from NCBI (Y14203) with a restriction enzyme sites for *Hin*d III and *Nco* I incorporated into the forward primer and *Bst*E II and *Kpn* I that was added through the reverse primer of Hind-HOXO primer set. The isolated barley *OXO* gene was digested with *Hin*d III and *Kpn* I and ligated into the *Hin*d III and *Kpn* I site of pGA1611 to generate pGA1611-OXO. The pGA1611-OXO was designed to carry and express the *OXO* gene into rice chromosome. This binary vector contained the hygromycin-resistance gene for selection of transformants and multiple-cloning sites within the transfer DNA (Lee *et al*., 1999). Plasmid DNA was isolated from the clones of pGA1611-OXO and sent for sequencing. The whole cassette which contains the ubiquitin promoter, barley *OXO* gene and Nos terminator was then digested from the pGA1611 with *Cla* I and *Bam* HI and ligated into *Sma* I and *Bam* HI of pCambia1301 to generate pCambia-MyOXO. The sequence verified pCambia-MyOXO was transformed into *Agrobacterium tumefaciens* LBA4404 and used for the rice calli transformation thereafter.

Tissue culture and rice transformation: The immature embryos of MR81 were collected from the sterile immature seed and sterilised with 20% Clorox followed by 1% zircon. The immature embryos were then washed with copious amounts of water. The sterile immature embryos were then cultured on the media MB at 25°C in the dark for 4 weeks (Murashige and Skoog, 1962). The immature embryos were sub-cultured every 3-4 weeks. The fresh calli which were cultured on new MS media for 5 days were co-cultured with *Agrobacterium* tumefaciens LBA4044 containing pCambia-MyOXO for 20 minutes, OD = 0.6 and the calli were then air-dried for 3 h on filter paper. The dried calli were then cultured on co-cultivation media at 25°C for 3 days. After 3 days of co-cultivation, the calli were washed with deionised water which contained 250 mg L^{-1} of cefotaxime. The calli were air-dried and cultured on selection media (4.42 g L^{-1} Murashige-Skoog premix media, 30 g L^{-1} sucrose, 500 μL L^{-1} Plant Preservative Medium (PPM), 250 mg L^{-1} carbenicillin, 100 mg L^{-1} cefotaxime, 35 mg L^{-1} or 50 mg L^{-1} hygromycin, 3.25 g L^{-1} agar, pH 5.7). The transformed calli were transferred to new selection media every 10 days.

The regenerated calli were cultured on the pre-regeneration media (4.42 g L^{-1} Murashige-Skoog premix media, 30 g L^{-1} sucrose, 0.1 mg L^{-1} NAA, 0.2 mg L^{-1} Kinetin, 5 mg L^{-1} ABA, 500 μL L^{-1} PPM, 250 mg L^{-1} carbenicillin, 50 mg L^{-1} hygromycin, 3.25 g L^{-1} agar, pH 5.7) for 10 days and then followed by regeneration media (4.42 g L^{-1} Murashige-Skoog premix media, 30 g L^{-1} sucrose, 0.5 mg L^{-1} NAA, 3.0 mg L^{-1} Kinetin, 500 μL L^{-1} Plant Preservative Medium (PPM), 150 mg L^{-1} carbenicillin, 50 mg L^{-1} hygromycin, 3.25 g L^{-1} agar, pH 5.7). The calli were cultured at 25°C under 12/12 light/dark regime for 3 months.

Screening of transformed plantlets: Transformed calli were selected on hygromycin selective media. Callus with no sign of growing is considered no transformation occurred and will be eliminated. Subsequently, the

positive transgenic plants after regeneration will further confirmed with PCR and Southern Blot assays (Southern, 1975).

RESULTS AND DISCUSSION

Amplification and identification of *OXO* gene: The barley *OXO* gene was amplified with a pair of primers designed using the barley OXO cDNA sequence from NCBI. The forward primer is 5'-AGCTTAGCAGCAACCAC CAG-3' and the reverse primer is 5'-AGCAATACA TTTTAAGTCCTCGCATT-3'. The amplified PCR product which is about 1 kbp in size (data not shown) was cloned and sequenced. The sequence was then verified via BLASTn and BLASTx from NCBI database (http://www.ncbi.nlm.nih.gov/). The BLASTn results showed the amplified gene has 100% similarity with barley oxalate oxidase HvOXOa sequence. The BLASTx results also showed that the translated protein sequence from the amplified sequence is similar with barley oxalate oxidase germin protein (EC 1.2.3.4) with 98% similarity.

***In silico* characterisation of *OXO* gene:** The amplified *OXO* gene is comprised of 1 exon with no intron with 224 amino acids from start to stop codon. The molecular weight and amino acid composition of OXO was analysed using ProtParam programme from ExPasy Proteomics Server (http://kr.expasy.org/tools/protparam.html). The predicted molecular weight for OXO is 23.6 kDa with an isoelectric point of pI 6.0 as predicted by Zhou *et al.* (1998). Meanwhile, the amino acid composition of OXO showed that glycine (11.2%) was the most frequently present and highly conserved amino acid in homologous protein sequences (Branden and Tooze, 1991). A total 47.3% of amino acid of OXO was hydrophobic amino acids, 25.3% was polar amino acids and 16.1% was charged amino acids. Thus, OXO is most likely a trans-membrane protein due to the high content of hydrophobic amino acids. SMART (http://smart.embl-heidelberg.de/) and TMHMM2.0 programmes were used to predict the presence of trans-membrane domains in the OXO Open Reading Frame (ORF). Results showed that the presence of trans-membrane signalling protein existed between amino acid residue 7 to 24 for SMART and amino acid residue 7 to 29 for TMHMM2.0. Graph of trans-membrane domains topology versus position of protein was sketched (Fig. 1) and the results show that OXO consists of one signalling peptide at N-terminus with high probability value. Further analysis was conducted with the SignalP programme (SignalP 4.0 from www.cbs.dtu.dk/services/SignalP/) to predict the presence of signal peptidase cleavage site and signal

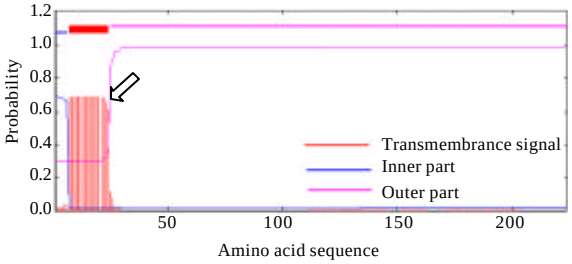

Fig. 1: Transmembrane protein analysis using program TMHMM2.0. Part of the blue protein is predicted protein in the cytoplasm while the purple represents the transmembrane signaling protein out of the cell, The arrow indicates the signal peptide

peptide in ORF of OXO. The data showed that signal peptidase was present at amino acid residue 1 to amino acid residue 23. The signal peptidase cleavage site was located at amino acid residue 23 and 24; it is predicted to be involved in the formation of mature protein. This reveals that OXO protein is contains N-terminal secretion signal peptide with approximately 23 amino acids pre-sequence. Thus, OXO is a secretory protein where it will be targeted into the cell wall and apoplast which may response in plant defence (Davidson *et al*., 2009; Druka *et al*., 2002). The length of OXO signal peptide is within the average range of the eukaryotic signal peptide which are 22.6 amino acids (Bendtsen *et al*., 2004).

Superfamily software was then used to determine the OXO protein family. The RmlC-like superfamily cupins was detected within the protein with a significant E value of $1.2e^{-40}$. RmlC-like superfamily cupins has 12 families; where germin or 7s seed storage protein is one of the families. In Germin family, there are four subfamilies which are germin, auxin binding protein, 7s seed storage proteins and oxalate decarboxylase (Murzin *et al*., 1995). The OXO protein was then analysed by Pfam PRINTS software which showed that OXO is a bicupin protein with significant E value, $5.1e^{-41}$ for cupin1 and $3.2e^{-11}$ for cupin2. The protein fingerprints analysis of PRINTS software showed that OXO sequence has a significant similarity with the germin family compared with the other ten protein families with a significant P value of $7.8e^{-28}$. The presence of three germin motifs in OXO nucleotide sequences with high similarity strengthens the identity of the OXO as a germin oxalate oxidase. Motif 1 is compatible with motif $G(X)_5HXH(X)_{3\text{-}4}E(X)_6$ whereas motif 2 matched the motif $G(X)_{4,5}PXG(X)_2H(X)_3N$ in germin. Analysis of OXO sequence using PROSITE software showed the presence of 11 conserved sequence motifs

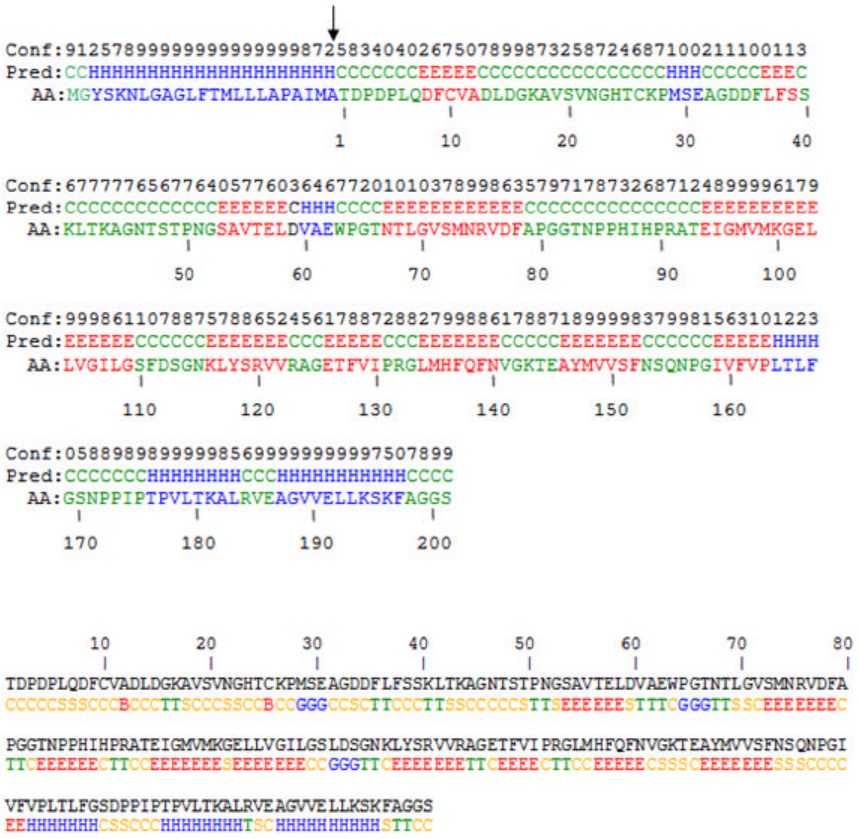

Fig. 2(a-b): (a) MyOXO protein secondary structure prediction by PSIPRED program. Numbering of amino acid residues from the mature protein after blocking peptidase site is indicated by arrows. Number 0 represents the lowest level of confidence (conf) while number 9 represents the highest level of confidence. E represents β-tail and C represents the loop (coil), H represents α-helix and (b) Secondary structure of the protein template 1F2I., H represents the α-helix, G represents helix-3_{10}, E represents β-tail and C representing the loop (coil), B represents the peptide bond, T represents the rotation (turn), S represents the arc (bend)

that from germin site, phosphorylation casein kinase II motif site, phosphorylation protein kinase C motif site, glycolate-N motif site and myristoylation-N motif site.

OXO secondary structure was predicted by using PSIPRED program and the results showed that there are 10 elements of β-sheet secondary structure and 6 α-helical forms (Fig. 2). 1st secondary α-helical structural element is 21 amino acids in length and it is a signal protein but it's not involved as a functional protein. OXO protein domain is a β-barrel jellyroll protein (Druka *et al*., 2002) that contains two pieces of β-sheet element, each of β are likely a β-tail as shown in the crystal structure of the germin domain (oxalate oxidase), 1FI2. Comparison between the secondary structure of OXO sequence and the 1FI2 template sequence showed that the 1FI2 sequence has an excessive helix 3_{10}. Presence of helix 3_{10} is unusual and is not involved in protein function (Branden and Tooze, 1991). Thus, the existence of a helix 3_{10} will not have a serious effect on the function of the OXO protein. Helix-α in the protein is predicted to be involved in the structural stability of the protein and serves as a key to grasps the dimer structure (Woo *et al*., 2000). Such interactions are hydrophobic and therefore are expected to provide high stability in the germin protein. Whilst, the β-barrel structure of protein is not geometrically stable and can be distorted. However, from the multiple sequence alignment between 143 sequences (data not shown) through BLASTp using NPSA; the germin and OXO protein sequences contains Cys^{68} and Cys^{91} residues in the N-terminal region. These two conserved amino acids might form the disulfide bond which wrapped around the β barrel where in turn creating

OXO protein in a β-barrel jelly roll like structure. This structure is essential to assist OXO protein structure which is interlocked with cofactors that have extreme resistance to heat and degradation. The cofactor binding with ligands in OXO protein's active site is with mononuclear Manganese, Mn(II) and none of the other metal-ion cofactors (Requena and Bornemann, 1999). It may take role in conversion of O_2 to H_2O_2 during oxidase enzymatic processes. With 3D ligandsite prediction software, one Glu (Glu^{118}) and three His ($His^{111,113,160}$) residues in OXO protein sequence is showed act as ligand for the binding of Mn(II) ions at the active site of OXO. The $His^{111,113}$ residues together with Glu^{118} are located within highly conserved germin motif 1, where His^{160} residue is in motif 2. This data corresponded with the findings of Gane *et al.* (1998) and Requena and Bornemann (1999) where four manganese ions were found binding to the cupin of germin. These four ligands are predicted to be used in trapping the free radicals during biological processes and catalytic activities.

Prediction of OXO protein 3-dimensional (d.) model: A total of 147 sequences were used in alignment analysis with protein database using BLASTp in the NPSA (Network Protein Analysis seq) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). The 3D structure of the OXO protein with labelled active sites is shown in Fig. 3. The multiple sequence alignment showed that the protein sequence contained. These residues were proposed by Woo *et al.* (2000) to be involved in the forming of disulfide bonds and as presenting a stabilising effect on the protein structure in the N-terminal region. IF2I, one of the 147 protein sequences used in the d. modelling of *OXO* has a 3-dimensional structure that was predicted through X-ray crystallography experiments (Woo *et al.*, 2000). The OXO amino acid sequences has 99% similarity with the protein structure of barley oxalate oxidase, 1FI2, a member of the subfamily oxalate decarboxylase through the multiple sequence alignment analysis (http://ca.expasy.org/tools/sim -prot.html) (Fig. 4) in the Expasy database software. In addition, the gap frequency between these two protein sequences is 0.0%. There are two different amino acid residues in these two sequences which is the residue of F at the amino acid residues N-135 and the amino acid-N at position 194 in the OXO sequence. Both residues are located in the loop and are likely not to affect the protein structure. The OXO protein

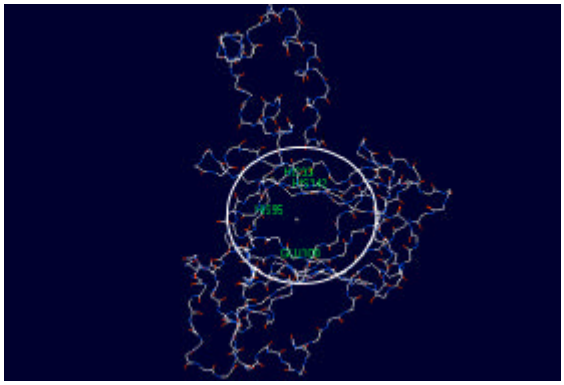

Fig. 3: d. structure of OXO protein Struktur 3D His^{160}, His^{162}, Glu^{167} and His^{214} are the active sites. White circle indicates the β-barrel domain

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99.0% similarity in 201 residues; Score: 1039.0; gap: 0.0%

MyOXO,      24 TDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLTKAGNTSTPNGSAVTELD
1FI2,        1 TDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLTKAGNTSTPNGSAVTELD
               ************************************************************


MyOXO,      84 VAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGMVMKGELLVGILGSFDSGNKLYS
1FI2,       61 VAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGMVMKGELLVGILGSLDSGNKLYS
               ************************************************** ********

MyOXO,     144 RVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQNPGIVFVPLTLFGSNPPIPTPVLT
1FI2,      121 RVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQNPGIVFVPLTLFGSDPPIPTPVLT
               ************************************************* *********

MyOXO,     204 KALRVEAGVVELLKSKFAGGS
1FI2,      181 KALRVEAGVVELLKSKFAGGS
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Fig. 4: Mature protein sequence alignment with the target template sequence with the SIM software, *Identical residues

structure was predicted using the 1st approach model in SwissModel (http://swissmodel.expasy.org/) website and the resulting protein structure is shown in Fig. 5. Comparison between OXO protein models with the protein template 1FI2 was further conducted by using combinatorial program Extension (CE) to measure similarity between both protein structures based on the distance of the carbon-α. Results showed that the Rmsd is small; 0.1 Å and therefore it can be assumed that OXO protein has similar structure to the template protein 1FI2.

Construction of Ubi-OXO-nos cassette: The barley *OXO* gene for cloning purpose was amplified with a new pair of primers designed base on the sequence of the amplified OXO from BAC clone 450N4 with incorporation of *Hind* III and *Nco* I to the forward primer 5'-AAAAAGCTTCCATGGGTTACTCTAAAAACCTAGG GGCTGGCCTGTTCAC-3' and *Bst* EII and Kpn I to the reverse primer 5'-AAAAAGGTGACCGGTACCT TAAGACCCACCGGCGAACTTG-3'. This primer set was used to amplify a 675 bp PCR product from the BAC clone 450N4. The amplified *OXO* gene with the incorporated restriction sites was named MyOXO and was cloned into pGA1611 within the *Hin*d III and *Kpn* I restriction site (Fig. 6).

The Ubi-MyOXO-Nos cassette which contained the maize ubiquitin promoter was then digested from the pGA1161 with *Bam*H I and *Cla* I. The whole cassette size of 3116 bp (promoter 2016 bp, OXO 675 bp, NOS terminator 400 bp) was then transferred into the plant transformation vector, pCambia1301 which contains the GUS reporter gene to facilitate the screening process in rice. The new constructs was named pCambia-MyOXO. Digestion of this construct with a combination of *Sma* I and *Bam*H I produced a linear band with size 15 kbp which equivalent to the size of pCambia1301 (11849 bp) and Ubi-OXO-Nos cassette (~3 kbp) (Fig. 7). The pCambia-MyOXO DNA was transformed into *Agrobacterium tumefaciens* LBA4404 and used for rice transformation.

The binary vector, pCambia1301 was used as backbone in the construction of OXO-expression cassette. Maize ubiquitin promoter from pGA 1611 was used to drive the *OXO* gene expression in *Oryza sativa indica* MR81 constitutively. It is believed that the maize ubiquitin promoter increases the transgene expression in indica rice cultivar than the rice native ubiquitin promoter. The maize promoter can avoid homology-dependent gene silencing in rice transgene expression (Park *et al*., 2010). We used *Agrobacterium*-mediated transformation to generate OXO transgenic rice as this method reduces gene silencing, promotes stable transgene expression, has low genomic rearrangement in the transgene plant recipient and thus it has been routinely used to transform foreign gene into rice. However, there are several drawbacks for this *Agrobacterium*-mediated transformation. In cereals, the transformation rate varied with genotype. Rice genotype is one of the crucial factors in rice transformation (Khanna and Raina, 1998). Therefore, local cultivar of *O. sativa*, MR81 which is responsive to tissue culture was selected for

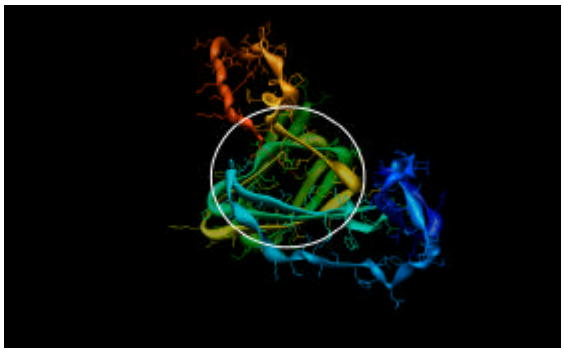

Fig. 5: MyOXO protein structure predicted by comparative protein modeling, β-barrel domain is in the white circle

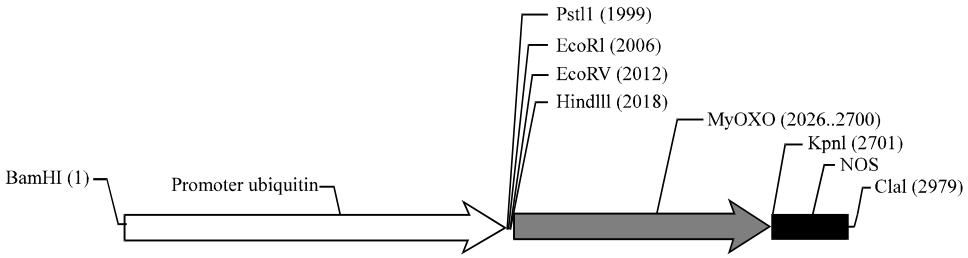

Fig. 6: Linear map of Ubi-MyOXO-Nos cassette. Ubiquitin promoter, MyOXO: Coding region of barley oxalate oxidase gene, NOS: Nopaline synthase terminator, 2984 bp

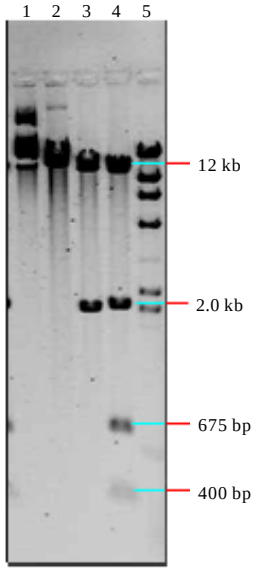

Fig. 7: Digestion analysis of pCambia-MyOXO construct. Lane 1: Undigested pCambia-MyOXO; Lane 2: pCambia MyOXO digested with *Sma* I and *Bam*H I; Lane 3 : pCambia MyOXO digested with *Bam*H I and *Hin*d III. Lane 4 : pCambia My*OXO* digested with *Hin*d III and *Kpn* I; Lane 5 : Lamda *Hin*d III-molecular marker Rice Calli Regeneration

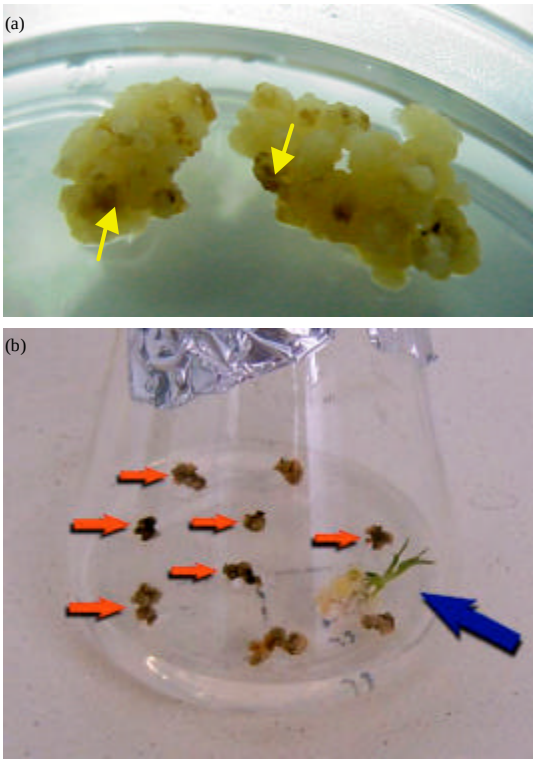

Fig. 8(a-b): (a) Calli in 5x magnification, (b) Regenerated calli after co-cultivation with *Agrobacterium*, Red arrow: Dead calli (in dark brown), Blue arrow: Regenerated calli which is resistant to hygromycin after transformation, Yellow arrow: Location where the calli begins to show emergence of plantlet

transformation purposes. In general, calli is the common explant for *Agrobacterium* mediated transformation, electrophoresis and also particle bombardment (De Clercq *et al*., 2002; Espinosa *et al*., 2002; Folling and Olesen, 2001; Manoharan and Dahleen, 2002; Wu and Feng, 1999; Christou, 1995). In this study, calli was induced with the immature seed on induction media (4.42 g L^{-1} Murashige-Skoog premix media, 30 g L^{-1} sucrose, 2 mg L^{-1} 2,4-D, 3.25 g L^{-1} agar, pH 5.7) at 25°C in dark for 4 weeks. The induced calli is shown in Fig. 8a.

Transformation of rice calli with the pCambia-MyOXO cassette: *Agrobacterium* transformed with pCambia-My*OXO* was co-cultured with the calli for three days in dark at 25°C. Acetosyringone at 100 µM concentration need to be added into the co-cultivation media and the culture temperature at 22-28°C is critical in order to produce a transformation at high efficiency (Hiei *et al*., 1994). The transformed calli are resistant to the hygromycin and regenerated into plantlet (Fig. 8b). The transformation efficiency for *Oryza sativa indica* cultivar MR81 is 1.7%. Present results demonstrated low transformation efficiency in the indica as compared to what has been reported with japonica and others rice cultivar. We observed browning and shrinking of indica rice calli in most transformation processes which may be a plant defense response after infection of *Agrobacterium* as reported by Tie *et al*. (2012). Following transformation with the *OXO* cassettes, the incorporation of the OXO transgene was validated and confirmed using southern blot and PCR amplification.

Molecular characterisation of transformed plantlets: We conducted a PCR analysis on five putative transformants. The PCR showed positive results for transformants T2, T3, T4 and T5 (Fig. 9). The four transformants showed a band that was approximately 700 bp in size which is the estimated size of the gene. Based on the results of the PCR, we concluded that the transformation of pCambia-MyOXO

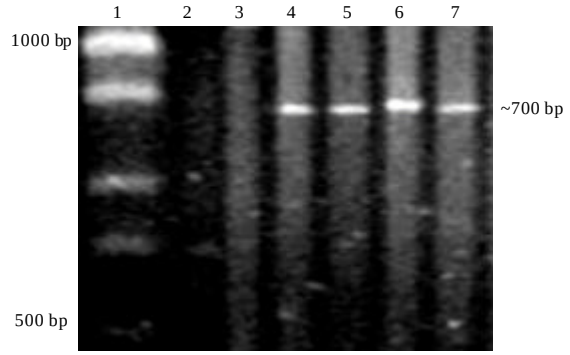

Fig. 9: PCR amplification of *OXO* gene in the putative transformants. Lane 1: 2-Log DNA Ladder, Lane 2: non-transformant, Lane 3: Putative transformant T1 (genomic DNA of MR81), Lane 4: Amplification of transformant T2, Lane 5: Amplification of transformant T3, Lane 6: Amplification of transformant T4, Lane 7: Amplification of transformant T5

into MR81 was successful. The T1 generation will be further tested to confirm the stability of the transformation.

CONCLUSION

In summary, barley *OXO* gene was successfully transformed into *Oryza sativa* cv. *indica* MR 81. The study found that *Agrobacterium*-mediated transformation was able to deliver the foreign gene into indica rice. The potential of OXO transgenic rice line is to generate elevated levels of OXO and hydrogen peroxide and thus provide wide spectrum defence against pathogen infestation will be tested against all rice pathogens. Once this has been determined, these lines may either be used as donors in conventional breeding, made available directly in the market as a rice variety or to be used in further transgenic studies to incorporate other resistance or defence related genes.

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