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MOLECULAR CLONING AND TRANSCRIPT PROFILING OF ASCORBATE OXIDASE GENE AT DIFFERENT GROWTH DEVELOPMENTAL STAGES FROM THERAPEUTICALLY IMPORTANT PLANT SEABUCKTHORN *HIPPOPHAE RHAMNOIDES* L.

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Abstract

Ascorbic acid (vitamin C) is a well-known molecule for its nutritional importance. However, the major aspects of its metabolic processes as well as a few of its functions in plants are poorly understood. A candidate gene *AO* (*Ascorbate oxidase*) known to be involved in ascorbate biosynthesis and metabolism was therefore chosen in seabuckthorn (*Hippophae rhamnoides* L.). In this study, full length cDNA sequence of *Hr-AO* was amplified and cloned through RT-PCR. The amino acid residues encoded by *Hr-AO* (2160 bp) were 719aa. There was a difference in length of newly isolated cDNA sequence as compared to tomato cDNA with 87% gene homology. Gateway cloning technique was also used to transfer *Hr-AO* gene to expression vector for functional study. Expression analysis of this gene sequence from six different tissues including vegetative bud, seed, shoot apex, green leaves, green fruit and mature (orange red) fruits showed maximum transcript accumulation in green leaf and young green fruit tissues. This is the first report on description of relationship between expression of *Hr-AO* and fruit development in such type of bush plant. This new gene isolated from seabuckthorn will help to understand the regulatory role of this enzyme in ascorbic acid metabolism. The investigation suggested that this gene could merely contribute toward ascorbic acid function or may be specific for further genetic engineering of crops.

Key words: Seabuckthorn, Ascorbate, Gene expression, Vitamins, Biofortification.

Introduction

Vitamin C or ascorbic acid (ascorbate) is an indispensable nutritive element for living organisms. Vitamin C relates to numerous vitamers having activities of vitamin C. Ascorbate oxidase belongs to class of multi copper enzyme catalyzing the oxidation of ascorbic acid to dehydroascorbic acid. Ascorbate oxidase is a cell wall localize enzyme that utilizes oxygen for oxidation of ascorbate (AA) to the unstable radical monodehydroascorbate (MDHA) which quickly disproportionate to give dehydroascorbate (DHA) and ascorbic acid, and hence added to regulation of the ascorbic acid redox state (Vasileios *et al*., 2006). However, plants and most animal have the ability to synthesize ascorbic acid whereas L-gulono-1,4-lactone oxidoreductase enzyme is absent in humans which is requisite for the last stage in ascorbic acid synthesis. Because AsA cannot be stored in the body, the vitamin should be attained frequently from dietary origins. Fruits and vegetables constitute the main dietary sources of Ascorbic acid in humans and current reports suggested that better AsA consumption (from 60 to 200 mg/d) may impart health benefit (Carr & Frei, 1999; Levine *et al*., 1999).

Vegetables and fruits are important constituents of the daily diet which contribute carbohydrates especially dietetic fiber, vitamin and mineral to the body. Vitamin C is commonly observed in several fruits and vegetables (Deman, 1973). This is water-soluble having antioxidant property well known for health and suitable functions of the human body (Benzie, 1999; Davey *et al*., 2000). It controls several syndromes like scurvy and also has a tendency of preventing several contagious diseases, including viral and bacterial diseases. This is also essential for curing injuries, burns and cracked bones. This vitamin is necessary for the production of all connective tissues (Heimann, 1980). Additionally the foods ample in fresh fruits and vegetables are defensive against chronic, degenerative diseases (Joshipura *et al.*, 1999; Lampe, 1999; Cox *et al*., 2000).

Ascorbate (AA) is the richest antioxidant found in plants and contribute mainly to cell redox state (Smirnoff, 2000). The largest part of the AA is contained in the cytoplasm; almost 10% of the AA contents of the total leaf is transferred and localized in the apoplastic region, where it is present in millimolar concentrations (Noctor & Foyer, 1998). Apoplastic ascorbic acid is supposed to characterize firstly protection against extraneous oxidants causing potential damage. These also have a significant function to mediate reaction to stress rendering an improved oxidative load (Barnes *et al.*, 2002; Pignocchi & Foyer, 2003). The organization of both reduced and oxidized types of ascorbic acid is shown in Fig. 1.1.

Oxidative damages are received by plants in case of both natural abiotic stresses all around their life-time and through terminal development as the final stage of fruit ripening senescence. It usually accompanied the last phase of maturation and thought to be partly induced by decreased effectiveness in fruit protection system against oxidation.

Plants oxidative defense mechanism mostly required the biosynthesis of antioxidant compound and stimulated expression of antioxidant enzymes e.g. catalyses, superoxide dismutases, ascorbate peroxidases (Felton & Summers, 1993; Halliwell & Gutteridge, 1989). The role of vitamin C as a cofactor is also associated with its redox potentiality (Combs & Gerald, 2012; Erdman *et al.*, 2012).

The seabuckthorn *Hippophae* rhamnoides ssp. *turkestanica* fruits have vitamin C substance in the range of 200 to 1500 mg/100g which is 5 to 100 times greater than any other fruit or vegetable (Ahmad & Kamal, 2002). Seabuckthorn berries have Vitamin C fluctuation due to geographic distribution where there is very little time for reproduction (Yao & Tigerstedt, 1995). The comparison of vitamin C among different species and varieties are shown in Table 1.1.

It is well known that fruits commonly comprise of great quantity of ascorbic acid and are an important supply of vitamins in food utilized by human (Davey *et al*., 2000).

Furthermore, these studies suggested that vitamin were being pointed as key enzymes of the biosynthesis pathway, modulating this regulation would seem to be a tangible approach to increase thiamin content in plants. Biosynthesis pathway need to be manipulated in order to increase vitamin levels for biofortification purposes in plants. Based on recent studies, it is now becoming clear that several regulatory steps will need to be taken into account in order to enhance the vitamin contents of staple crops.

The current study was attempted to appraise regulatory role of ascorbate oxidase gene in plant development, to isolate and clone ascorbate oxidase gene required in biosynthesis and metabolism of ascorbic acid and study levels of expression in different plant organs.

Materials and Methods

Wild seabuckthorn (*Hippophae rhamnoides* ssp. *sinensis*) berries were grown at the research station at National Institute for Genomics and Advanced Biotechnology (NIGAB) Islamabad. For nucleic acid extraction, the leaf, berries, bud and seed samples were harvested from plants at different developmental stages. Fruits were harvested once or twice a week during the ripening period from seabuckthorn nursery, frozen immediately in liquid nitrogen and placed at -80℃ until processed for nucleic acid extraction.

Designing of primers: Nucleotide sequences of ascorbate oxidase genes were retrieved from National Center for Biotechnology Information (NCBI) database. The gene specific primers were designed from the conserved region of ascorbate oxidase gene for the amplification of full length cDNA of *Hr-AO* gene. Primers for expression analysis through RT-PCR were designed from newly isolated gene sequences of *Hr-AO* gene. All these primer sequences are given in Table 1.2.

RNA isolation: The seabuckthorn fruits and leaves samples were homogenized in liquid nitrogen at room temperature. About 0.1 g of tissue was transferred into ice cold micro centrifuge tubes. About 500 µl RNA reagent was added, vortexed. It was incubated at room temperature for 5 min. For this period of incubation, micro centrifuge tubes were kept horizontally. The Samples were centrifuged for 2 min at 12800 rpm at room temperature, and supernatant was transferred to the new tube. 100 µl of 5 M Nacl was added and mixed for a while, and then 300 µl of chloroform was also added and mixed thoroughly. The samples were then centrifuged at 4ºC for 10 min at 12800 rpm. The uppermost aqueous phase was transferred into new micro centrifuge tube and equal volume of isoproponal was added, mixed and kept at room temperature for 10 min, centrifuged again for 10min at 4ºC at 12800 rpm. The RNA pellet was washed with 70% ethanol. The RNA was stored at -80ºC for RT-PCR experiments.

RT-PCR amplification of *Hr-AO* gene and purification: This good quality total RNA was used for the synthesis of cDNA with AMV-RT reverse transcriptase enzyme. The good quality seabuckthorn cDNA was utilized as template in RT-PCR for amplification of full length *Hr-AO* cDNA fragment. Total reaction of 50 µL volume was used with the following regents added: 5 µl of 10× Buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 0.5 µl of Taq Polymerase, 1.5 µl of 10 µM forward primer and 1.5 µl of 10 µM reverse primer.

The standard PCR was completed with gene specific primers under the following program: a primary denaturation step of 5 min at 94ºC, 35 cycles of 94ºC 60 s, 59ºC 60 s and 68ºC 165 s, followed by last extension step of 68ºC for 10 min. Furthermore, 1% agarose gel was used for the examination of amplicon and photographs were taken. These amplified fragments were gels purified and sequencing was carried out to validate the target sequence. The total PCR products was first run on 2% high resolution agarose gel and then purified by PCR GeneJET PCR purification kit (K0701).

The purification product was transported to MACROGEN (Korea) intended for sequencing. The purified gene was ligated into the TA cloning vector pTZ57R/T and was confirmed through PCR using M13 primers and sequencing.

Gateway cloning: This technique was used to efficiently transfer *Hr-AO* DNA fragment among plasmid vectors. This was accomplished by the use of recombinant sequence set of "Gateway att" site and two enzyme mixes, named "LR and BP Clonase". We successfully transferred gene fragment among various vectors used for cloning. Initially, the gene fragments were first inserted in plasmids having both adjoining recombinant sequences att L 1 and att L 2 in order to create a Gateway Entry clone. These Entry clones were generated in two steps.

Gateway entry clone: *Hr-AO* gene product was amplified by using Taq polymerase in PCR reaction with gate way primers designed (Table 1.2). The recombinant plasmid was used for GW product amplifications using same PCR procedure. GW product were amplified with specific Gateway primers by using program: a first denaturation cycle at 94ºC 3 min, 35 cycles at 94ºC 60 s, 57ºC 75 s and 72ºC 165 s, followed by last extensions step at 72ºC for 10 min. Amplified products were examined on 1% gel and photographs were taken. Low melting agarose gel was used to purify the total PCR products which were ready to use for BP reaction.

Table 1.1. Comparative list of the Vitamin C content in seabuckthorn berries, seed oil & pulp.

Vitamin	Species/Varieties	Contents			Reference
		Berries/Juice	Seed &oil	Berries Pulp	
Vitamin C (mg/100g)	European subsp. *rhamnoides*	360–2500	–	–	Yao *et al*. (1992), Zeb (2004a)
	Pakistani SBT	250–333	–	–	Sabir, Maqsood, Ahmed *et al*. (2005)
	Pakistani SBT	150–250	–	–	Sabir *et al*. (2003)
	Pakistani SBT	263.05-399	–	–	Shazia *et al*. (2010)
	Pakistani SBT	191-295.6	–	–	Asad *et al*. (2007)
	European subsp. *rhamnoides*	28–310	–	–	Yao *et al*. (1992), Rousi & Aulin (1977)
	Fluviatilis subsp.	460–1330	–	–	Darmer (1952)
	Chinese subsp. *sinensis*	200–2500			Zheng & Song (1992), Yao *et al*. (1992)
	Chinese subsp. *Sinensis*	460–1330	–	–	Yao *et al*. (1992)
	Finnish SBT	29–176	–	–	Tiitinen *et al*. (2005)
	Subsp. *sinensis*	200–780	–	–	Zheng & Song (1992)
	Subsp. *sinensis*	600–2500	–	–	Yao *et al*. (1992)
	Subsp. *rhamnoides*	165.7–293.3	–	–	Rousi & Aulin (1977)
	Subsp. *rhamnoides*	150–310	–	–	Darmer (1952)
	Subsp. *rhamnoides*	27.8–201	–	–	Yao *et al*. (1992)
	Subsp. *mongolica*	40–300	–	–	Plekhanova (1988)
	Indian SBT	168.3–	–	223.2	Arimboor *et al*. (2006)
	Indian SBT	509	–	–	Katiyar *et al*. (1990)
	Indian SBT	422–416 –	–	–	Chauhan *et al*. (2001)
	SBT	360–2500	–	–	Li & Schroeder (1996)
	Chinese SBT	513–1676	–	–	Zhang Yan *et al*. (1989)
	Chinese SBT	780.0	–	–	Mingyu *et al*. (2001
	Turkestanica SBT	200–1500	–	–	Ahmad & Kamal (2002)
	Turkestanica SBT	–	35.4	–	Zeb & Malook (2009)
	Portland SBT	114–1550	–	–	Dharmananda (2004)
	Chinese SBT	300–1600	–	–	Xu (1956), Tian (1985), Wang (1987)

*Sea buckthorn (SBT)

Table 1.2. Detail of primers used for *H. rhamnoides AO* cDNA cloning, expression analysis (RT-PCR) and Gateway cloning.

Gene	Primer name	Primer sequence (5'-3')	Product size (**bp)
AO	AO-S	ATGGTTGAGCATGATTTTCATCAACAAA	2160bp
	AO-A	TTATAGAATTTAAGGCCTGTGGAA	
Actin-1	Actin-F	GAAGCCTTTGGACCCGTTTTT	290bp
	Actin-R	ACGAGGACGACCCACAATAC	
AO	AO-F	GAGGCAGACGGTCACTATGTGG	480bp
AO	AO-R	TCGAAAGCATGTAACAAGTTGTGT	
AO	AO-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTA TGGTTGAGCATGATTTTCATCAACAAA	2160bp
AO	AO-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCT TAAGGCCTGTGGAACCTTTTACTTT	

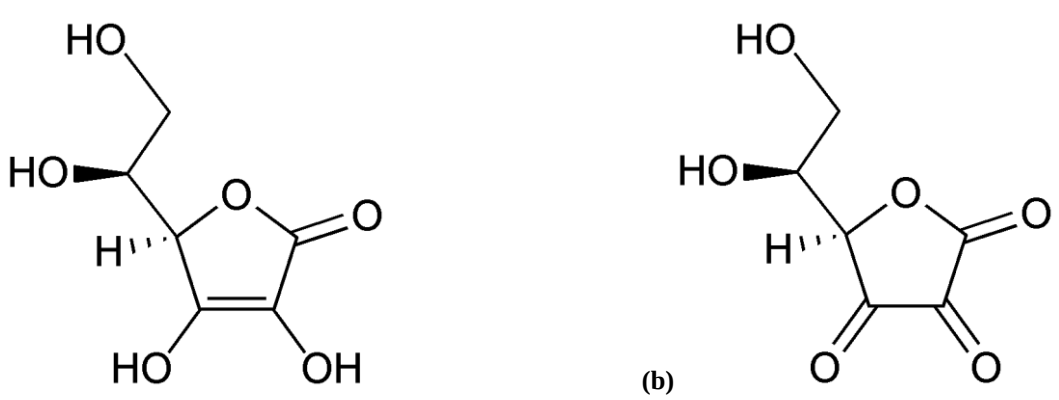

Fig. 1.1 Structure of ascorbic acid (a) ascorbic acid (reduced form) (b) dehydroascorbic acid (oxidized form).

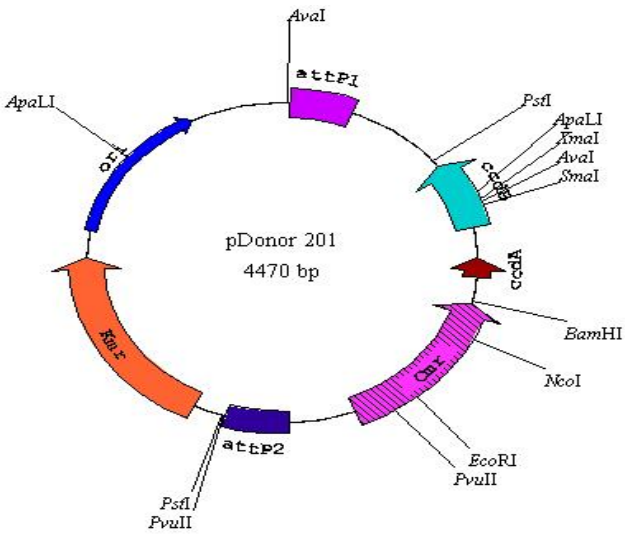

Fig. 1.2. The map of pDONAR™201 vector used in gateway cloning.

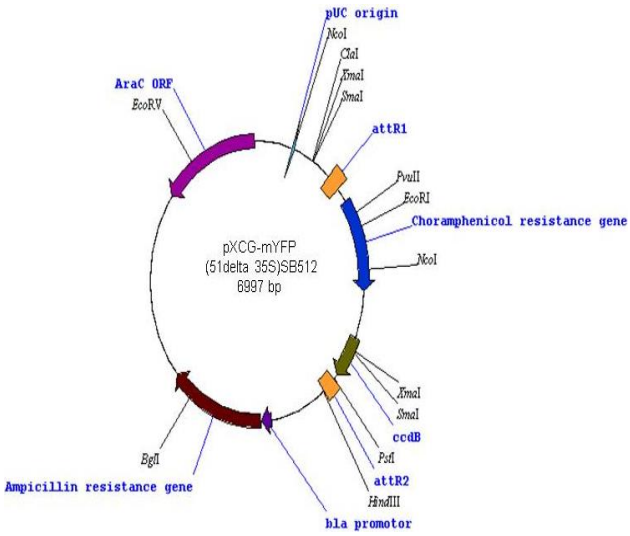

Fig. 1.3. Map of the destination vector pXCG-mYFP (51delta 35s) SB512 used in Gateway cloning.

BP cloning reaction: The BP reactions was set up using the pDONAR™ 201 vector (1 µl), fresh gateway PCR product **(**1-7 µl) and 8 µl of TE Buffer (pH 8.0). The map of pDONAR™201 vector was constructed in Sim Vector 4.6 software as shown in Fig. 1.2. These components were mixed carefully on ice in a 1.5 ml centrifuge tube and incubated at room temperature for 5 min. After thawing BP Clonase™ II enzymes on ice mixed it for about 2 min. Then 2 µl of BP Clonase™ II enzyme was added to the reaction mixture by vortexing briefly twice and centrifuged briefly. The reactions were then incubated for 1 hour at 25°C. These samples were placed on ice before proceeding to transformed electro-competent *E. coli* cells.

Single vial of *E. coli* cells (DH5α) were thawed resting on ice. About 2 µl of the BP mix was poured in vial of electro-competent *E. coli* (50 µl) cells and was mixed carefully. It was incubated for few min lying on ice and transferred to pre cold cuvette. The cells were then electroporated at 1800 volts for transformation of pDONAR™ 201 vector into DH5α cells without shaking. These tubes were transferred on ice immediately. About 500 µl LB liquid media was added, mixed gently by pipetting and transferred into sterile micro centrifuge tubes. It was incubated for 1 to 3 hours by temperature of 37°C through continuous agitating. The bacterial cultures (250-300 µl/plate) were spread on a pre-warmed LB agar plate which contained kanamycin (100 µg/ml). Kanamycin was used as suitable marker for selection of donor vectors. These plates were incubated at 37°C overnight. All transformed colonies produced white color competently. The positive colonies were also grown further by striking on separate kanamycin resistant LB plate. The well grown colonies were selected and colony PCR was performed to confirm the transformants with gene specific primers. The colonies were used to inoculate 5 ml LB liquid media (added 5 µl kanamycin) and were grown overnight. This culture was used to purify plasmid for further reaction and sequencing. The BP purified plasmids were also confirmed through PCR with gene specific primers. The BP plasmids were diluted and PCR reaction was performed following the same PCR profile as used for colony PCR.

The purified plasmids were sequenced from Macrogen Korea and were confirmed for the *Hr-AO* gene with gene specific primers. This entry clone was now prepared for entry into destination vector.

LR reaction: LR reaction was used to transfer *Hr-AO* gene from Gateway entry clone into destination vector. This was easier method and following components 1-7 µl of entry clone, 1 µl of destination vector and 8 µl of TE Buffer (pH 8.0) were added using the same order at room temperature. LR Clonase™ II enzymes was thaw on ice and were vortexes and mix it shortly two times. About 2 µl of LR Clonase™ II enzyme mix was poured into the reaction and mixed by vortexing briefly and then centrifuged. This reaction was incubated at 25°C for 1 hour. The map of destination vector used is shown in Fig. 1.3.

The same protocol was followed as designated for BP reaction for transformation, with the exception that we used appropriate selection markers for the LB plate suitable for our destination vectors (typically 100 µg/ml ampicillin) instead of kanamycin. The total LR reaction was used for transformation and plating. The transformed cells were analyzed by colony PCR and positive colonies were marked and grown for plasmid purification. The construct was ready to use for further sequencing, cloning and expression process.

***AO* gene expression analysis:** The semi-quantitative RT-PCR amplifications were used to differentiate expression pattern of *AO* gene from seabuckthorn plant. Different tissues including vegetative buds, fresh seeds, shoot apex, green leaves, young green fruits and mature orange red fruits were collected from the plant grown in the glass house at the NIGAB Islamabad. Samples were instantly frozen using liquid nitrogen and were utilized for total RNA isolation with Trizol reagent. The RNA was treated with rDNAse to remove any DNA contamination and was quantified through Thermo Scientific's NanoDrop™ Lite spectrophotometer. 1.5% agarose gel was used to evaluate the total RNA quality.

The *AO* gene's primers were designed in NCBI primer picking program. These primers were designed based on the sequence of new gene isolated. The cDNA synthesized from different tissues was utilized as template in RT-PCR reaction with the subsequent program: first cycle of denaturing at 95˚C 5 min, 37 cycles of 94˚C 30 s, 58˚C 30 s and 68˚C 30 s, following last extension step of 68˚C 10 min. *Actin-1* was utilized as control gene to check equal PCR loading and quality check. These reactions were repeatedly carried out thrice for both biological and technical replicates. The transcript products were examined on 1% agarose gel stained with Ethidium Bromide (EtBr). The gel documentation system was used to photograph the amplicon. The transcript accumulation was evaluated from band intensity.

Results

Isolation and sequence analysis of large fragment of *AO* cDNA from seabuckthorn: RT-PCR techniques were utilized for amplification of full length fragment of *Hr-AO* cDNA. The cDNA was synthesized from purified RNA using the reverse primer from the gene specific primer set AO-R 5' TTATAGAATTTAAGGCCTGTGGAA 3'. The same cDNA was applied for the amplification of PCR products of approximately 2.2 kb. we have successfully isolated and cloned enormously long fragment of *AO* gene from seabuckthorn that exactly match the target cDNA sequence. The length and purity of new cDNA was determined by the same sort of gel electrophoresis method and sequencing. This new cDNA amplicon has full size of 2160 bp as compared to 1737 bp size of reference gene from tomato. The distinct high molecular weight band of *Hr-AO* gene observed on agarose gel (Fig. 1.4) was purified and cloned. The positive clones, containing *Hr-AO* gene insert, were picked out from thousands of transformed cells by blue white screening. The colony PCR was carried out using M13 primers to confirm clone with inserts. Plasmid extraction was carried out with Favorprep™ plasmid DNA extraction Mini Kit. The plasmid containing 2.2 kb cDNA fragment of this gene were confirmed through PCR and sequencing. The new data of cDNA sequence was analyzed using (NCBI) Search System (www.ncbi.nlm.nih.gov/). The sequence similarity was searched by using BLAST program against EMBL plant DNA sequence and swissport protein database. The identity score of *Hr-AO* compared to some other plant *AO* sequences was commonly found in range from 60% to 87%. Comparatively the expected *Hr-AO* nucleotides and amino acids sequences of seabuckthorn plant with that of tomato plant *AOs* indicated high conservation in sequences.

Our sequence analysis implied that *Hippophae rhamnoides ssp. sinensis AO* genes encoded enzymes homological to *AOs* from different plants having similarities to other multicopper oxidases genes. Here we reported elaborate molecular study of ascorbate oxidase gene addressing differences in length and amino acid residues. It belongs to Cupredoxin super family.

Clustal W alignments from *Hr-AO* cDNA with reference gene sequence at both nucleotide and amino acid level were created and considerable variability was observed in the sequence and size of this newly isolated cDNA sequence. The deduced amino acids sequences from the nucleotide sequence indicated that product of translation was a precursor of about 719 amino acid residues with *E* value of 7.49E-82. The sequence identity was 87% at amino acid level.

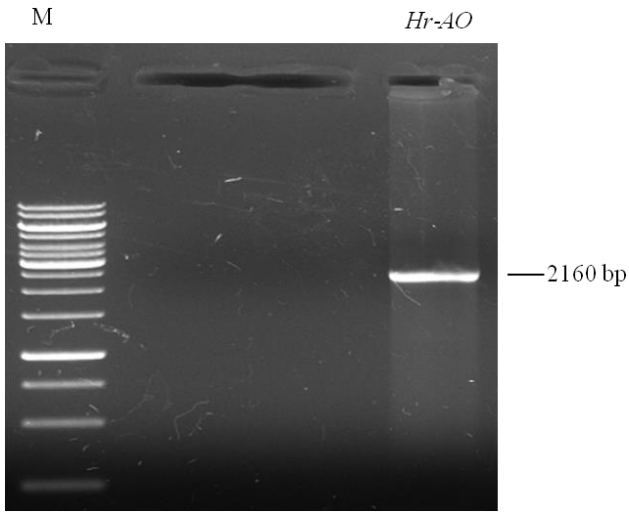

Fig. 1.4 (b) Amplification of Hr-AO gene using RACE technique of PCR. M = 1Kb DNA ladder.

Gateway cloning: In addition to the molecular cloning and sequencing Gateway cloning was carried out to transfer gene to expression vector. This is a robust scheme of creating an extensive range of expressions construct for function in manifold host system. The gate way ascorbate oxidase primers were used to amplify *GW-AO* from *Hr-AO* gene product as shown in Fig. 1.5a. Gate way cloning was used to easily shuttle our targeted sequence (*Hr-AO*) accompanying recombination with attB PCR products and attB expression clones. pDONR recombinant plasmids were purified and shown in Fig. 1.5b. The Entry clones were purified and sequence verified. *AO* gene specific primers were used to analyze entry clones by using PCR reactions. After creating entry clones, our concerned gene were transferable in great collection of expressions vector by means of Gateway® LR reactions involving entry clone and destination vector of our interest. This clone was ready to be used for further introduction and expression into the system of choice. Further the stable integration of *AO*-PB clones carrying the recombinant plasmid into destination vector was carried out. The pDEST vector pXCG-mYFP (51delta 35 s) SB512 recombinant plasmid were shown in Fig. 1.5c. The purified plasmids were further confirmed through sequencing. ClustalW multiple alignment of *GW-AO* sequence with original sequence was carried out using MultAlin tools as shown in Fig. 1.6. Due to efficient cloning the resultant construct were either screen or analyze separately, and were immediately applied in practical screening to observe better proteins variant.

Differential expression of *AO* gene among different tissues: In order to differentiate the expression pattern of ascorbate oxidase gene in various tissues of seabuckthorn, total RNA was extracted from six different tissues including vegetative bud, fresh seed, shoot apex, leaf, green fruit and mature fruit. This method involves RT-PCR amplification of gene transcripts using mRNA reverse transcription and a second step of semi-quantative

PCR amplification of the cDNA synthesis in comparisons with control actin gene. In our expression studies, the transcript signals of *AO* were strongly detectable in the green leaf tissues (Fig. 1.7). The transcript signals were also significant in green fruit tissues, whereas weak band intensity was found in vegetative buds and shoot apex. More interestingly no amplicon were detected in fresh seeds tissues. The overview of different *AO* genes, function and expression in specific tissues with their reference source was described in Table 1.3.

The ascorbate content in plants varies with different tissues and plant species. The ascorbate concentration in pepper is generally higher than that in tomato (Ogunlesi *et al.,* 2010), and the ascorbate content in leaves is much higher than that in fruits which support our findings. Photosynthetically active tissues in addition to fruit and other storage organs usually contain relatively higher concentration of ascorbate (Loewus & Loewus, 1987). Ascorbate content in rapidly-growing tissues is higher than that of aging tissues, as ascorbate is generally accumulated in the tissues with active growth such as the meristem (Luwe *et al*., 1993). This is consistent with the fact that content of ascorbate decreases with plant growth in most of parts of dill plants (Lisiewska *et al*., 2006). The ascorbate content in plants also varies with developmental stages (Birghila *et al.,* 2004). The ripening fruits in tomato accumulate more ascorbate than immature fruits. The ascorbate content is reported to be affected by plant physiological status and environmental factors (Smirnoff, 1996).

Our result also corresponds to gene expression studies in melon (Sanmartin *et al*., 2007). Gene-specific expressions work in melon plant indicated that simply *CmAO1* and *CmAO4* genes showed transcriptional activity and deferential regulation depending upon tissues, development stages in addition to stimulus by outside. Further they found *CmAO1* gene transcript in floral and fruits tissue, while *CmAO4* transcripts preferably accumulated in vegetative tissue without any expression of *CmAO* genes in seeds of melon. Although expression activity of *CmAO4* gene was observed during germination. Moreover regulation in activity of *CmAO4* as a result of stresses by heating, hormones and wound were also noticed (Sanmartin *et al*., 2007). These results corresponded to our expression studies in seabuckthorn plant where we have found *AO* expression activities in actively growing fruit and vegetative tissues.

Further reports of maximum expression of *AO* in cucurbits were found in different active growing parts of plant with considerable increase in activities and expression level of melon *AO* transcripts through the particular developmental stage of fruits (Esaka *et al*., 1992; Moser and Kanellis 1994; Diallinas *et al.,* 1997; Al-Madhoun *et al.,* 2003). (Moser & Kanellis, 1994; Diallinas *et al*., 1997).

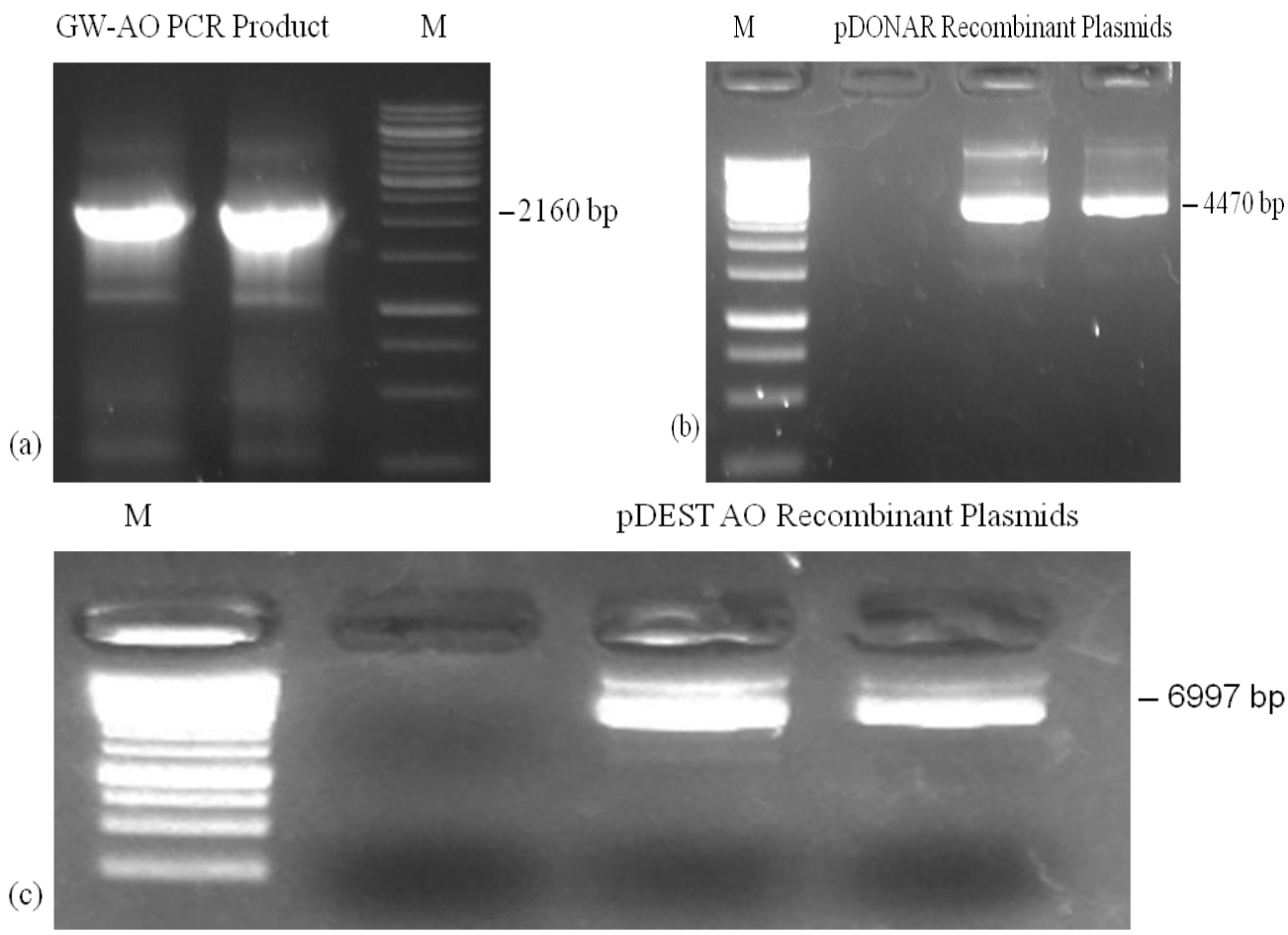

Fig. 1.5. (a) Amplification of GW-AO product by standard PCR with gateway primers. (b) Gel showing pDONAR recombinant plasmids. (c) The AO pDEST vector pXCG-mYFP (51delta 35s) SB512 recombinant plasmid constructs. M: 1 kb marker.

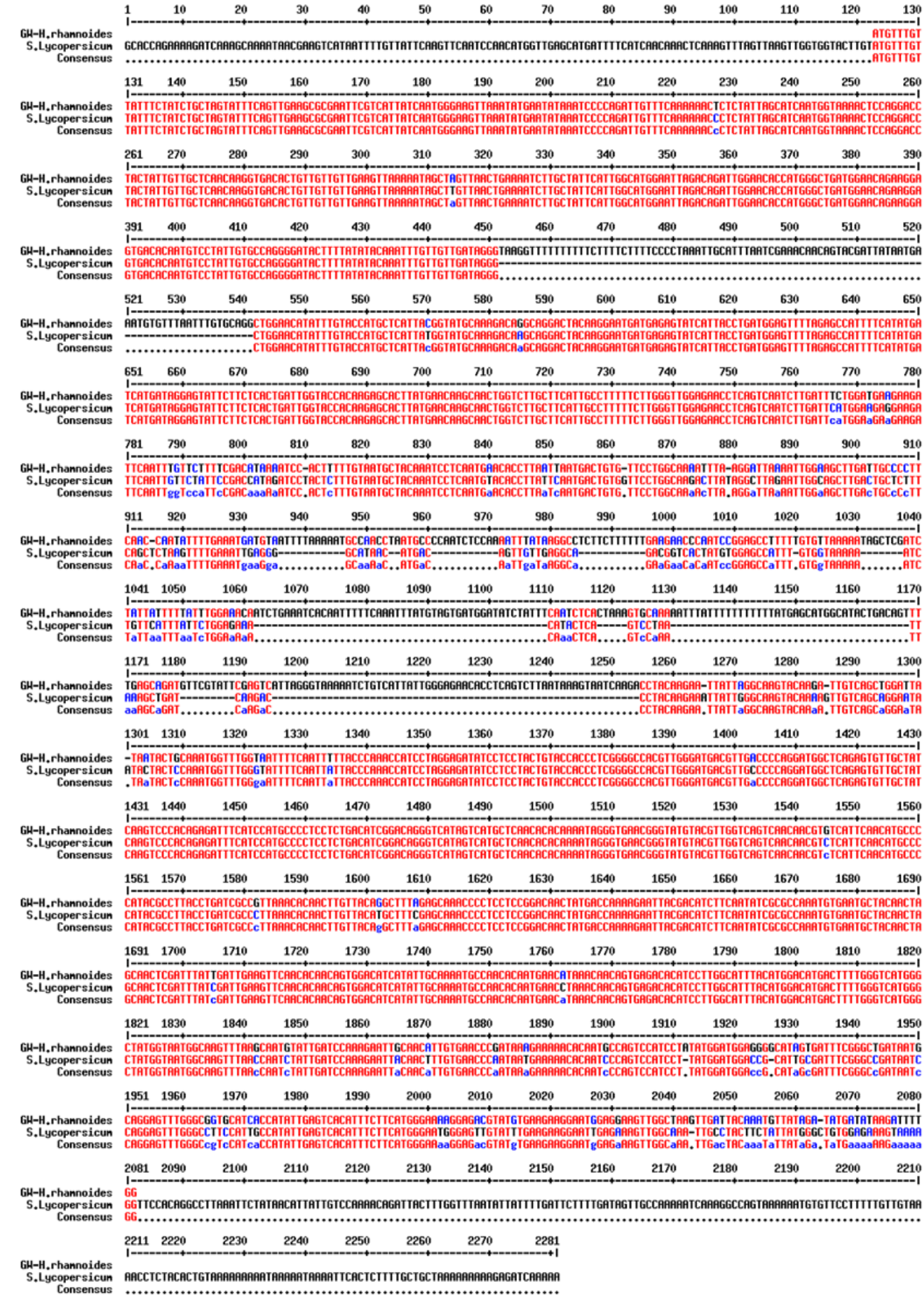

Fig. 1.6. ClustalW multiple alignment of *GW-AO* sequence with original sequence with online MultAlin *tools.* Strictly similar regions are highlighted against red color.

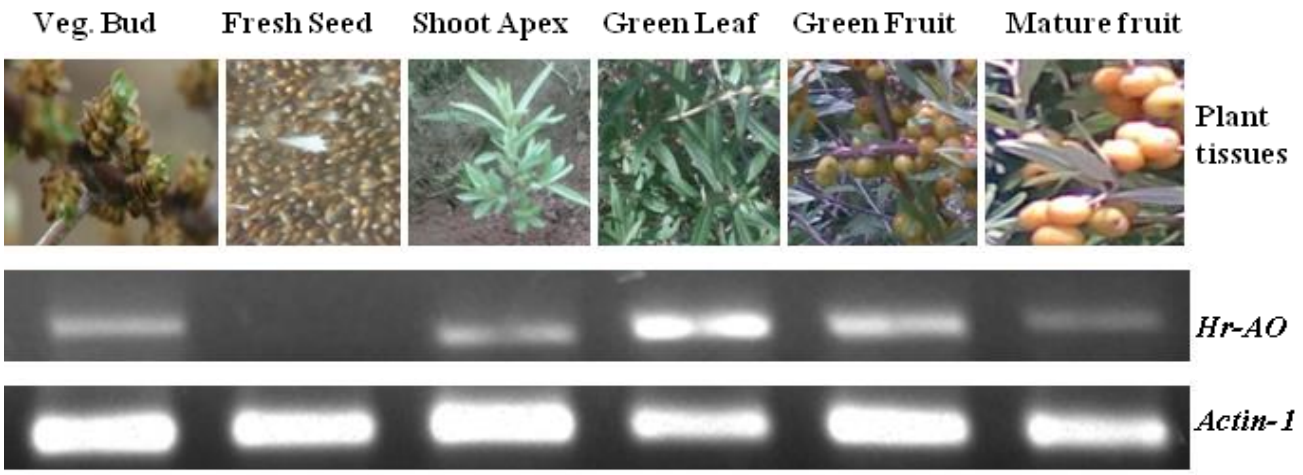

Fig. 1.7. *Hr-AO* transcript amplifications in vegetative bud, shoot apex, green leaf, green fruit and mature orange red fruit tissues of Seabuckthorn through semi quantative RT-PCR in comparisons with Actin gene. Actin gene is used as control, to check equal loading and PCR quality check. There was no band in case of seed tissues.

Table 1.3. Overview of different AO genes, function and expression in specific tissues with their reference source.

Lf	Fl	SP	Pt	St	Fr	Rt	Analysis	Function	References
/	/	/	/	/	+	/	RT-PCR	AA biosynthesis & metabolism	Zou *et al*., 2006
+	/	/	/	/	/	/	RNA and DNA Blot	Oxidation of AA to dehydroAA	Chou *et al*., 2004
+	/	/	/	/	/	/	Southern Blot	/	Naohiro & Muneharu, 1996
/	/	/	/	/	+	/	Fluorescence measurements	Blue copper protein	Eleonora *et al*., 2006
+	/	/	/	/	/	/	Genome sequencing & mapping	/	Tuskan *et al*., 2006
+	/	/	/	/	/	+	Northern RNA hybridization	/	Gamas *et al*., 1996
+	/	/	/	/	/	/	RT-PCR, RNA blot analysis	/	Sanmartin *et al*., 2003
/	/	/	/	/	+	/	RNA blot hybridization	/	Ohkawa *et al*., 1989
+	/	/	/	/	+	/	mRNA expression analysis	/	Li *et al*., 2010
+	/	+	/	/	+	/	Cloning & expression analysis	Induction & Repression of AO	Diallinas *et al*., 1997
+	+	+	+	+	+	+	Cloning and expression analysis	Growth & development	Yuanxiu *et al*., 2013
+	+	+	/	+	+	+	RAFL cDNA	/	Smirnoff, 1996
/	+	/	/	+	/	+	Northern & southern blot	Enzymatic activity	Garcia-Pineda *et al*., 2004
+	/	/	/	/	/	/	Northern blot analysis	Defense response	Barbehenn *et al*., 2008
+	/	+	/	+	+	/	Northern blot analyses	Regulation of AO expression	Esaka *et al*., 1992
+	/	/	/	/	/	/	PCR, Southern blot analysis	/	Pignocchi, 2003
+	/	/	/	/	/	/	Northern blot analysis	Defense responses	Hagihara *et al*., 2004
+	/	/	/	/	/	/	Cloning & RNA blot analysis	AO over-expression	Fotopoulos *et al*., 2006
+	/	/	/	/	/	/	Cloning and characterization	Up-regulation & resistance	Ren *et al*., 2013

Lf= leaf, Fl = flower, Sp= sepal, Pt= petal, St= stem, Fr= fruit, Rt= root, += expression and /= no expression

Our results are also in agreement with expression work in strawberry (*Fragaria×ananassa*) where high expression level of *FaAO* is found in more young parts (young fruits) with low expression in late maturing stages. It could be linked to the facts of active growth in young fruit tissues with important effects of *FaAO* on increasing fruits development. It may likely be due to reason of strong respirations as well as higher ethylene production within matured fruit, which suppressed *FaAO* gene expression in fruit tissues of strawberry (Yuanxiu *et al*., 2013). We can imagine that ascorbic acid contents in matured fruit are less which can be due to over-ripening of fruit with some degradation by enzyme then the activity growing leaf and green tissues. Moreover the transcript intensity was higher in green fruit tissues than the ripened fruit tissues. Leaf tissues showed the maximum transcript accumulation which showed maximum ascrobate oxidase accumulation and expression in apoplastic region.

Phylogenetic analysis: Phylogenetic analysis revealed that the *Hr-AO* was the member of the copper ion binding, oxidoreductase, family and it was assigned the unique name *Hr-AO*, alignments of the full length sequence of the *Hr-AO* and AY971876 (*AO*) showed 87% identity

In order to invoke an evolutionary model between newly isolated sequence and those in the database, phylogenetic reconstruction was carried out. Multiple sequence alignments were completed with Clustal W alignments using Mega 5.0 program and homology searches were done with BLAST programs in NCBI database. For this purpose the coding sequences of *AO* homologues were collected from various plants species including *Hippophae rhamnoides, Solanum lycopersicum, Ricinus communis, Glycine max, Medicago truncatula, Pisum sativum, Nicotiana tabacum, Populus trichocarpa, Oryza sativa, Arabidopsis thaliana, Laccaria bicolor, Brassica juncea, Cucurbita maxima, Cucumis melo, Cucumis sativus, Fragaria × ananassa, Malus domestica* and *Mentzelia*

pumila were used. The tree was built by majority decree and strict consensus. Terminal gaps were eliminated before analyzing the sequences. Whereas the inner alignments gaps were left and analyzed using scoring gap as character or as missing character. A tree was created using neighbor-joining method. 1000 bootstrap replicate were used for bootstrap analysis. Tree view was used to display resulting tree as shown in Fig. 1.8.

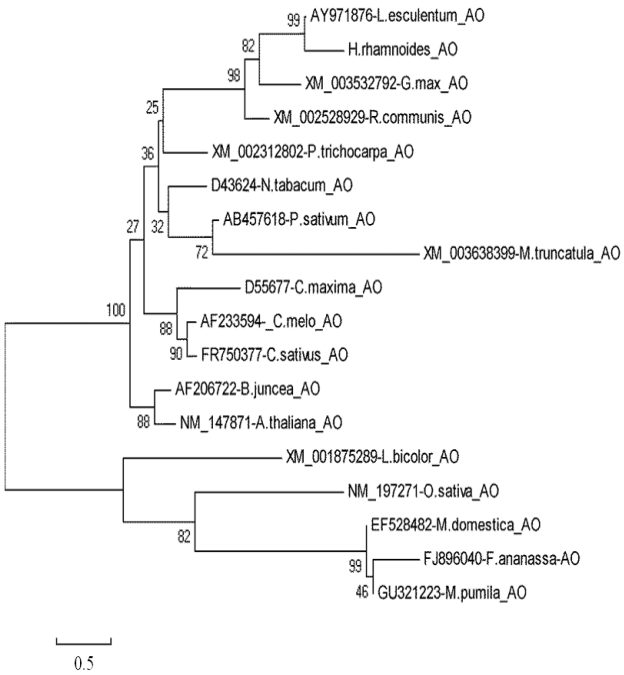

Fig. 1.8. Phylogenetic reconstruction of *ascorbate oxidase* gene from different species. Neighbor joining tree was made by means of MEGA5 software. The Value on each node indicated bootstraps replication of 1000. The species along with accession used in tree includes *H. rhamnoides-AO,* AY971876-*L.esculentum-AO,* XM_002528929-*R.communis-AO,* XM_003532792-G.*max-L-AO,* XM_003638399-*M.truncatula-AO,* AB457618-*P.sativum-AO,* D43624-*N.tabacum-AO,* XM_002312802-*P.trichocarpa-AO,* NM_197271-*O.sativa-AO,* NM_147871-*A.thaliana-AO,* XM_001875289-*L.bicolor-AO,* AF206722-*B.juncea-AO,* D55677-*C.maxima-AO,* AF233594- *C.melo-AO,* FR750377-*C.sativus-AO,* FJ896040-*F.ananassa-AO,* EF528482-*M.domestica-AO and* Gu321223-*M.pumila-AO.*

This tree displayed that sequences were evidently differentiated into different clades with five different clusters. Higher bootstrap values are indicative of increased reliability of the tree. Fascinatingly divergence in sequences was found with *H. rhamnoides* sequence lay close to *Solanum lycopersicum*, then close to *G. max, R. communis* and some other members of the Fabaceae family. This is very outstanding discovery that *Hr-AO* along with solanaceae member showed close relation with Fabaceae member where AOs were strongly associated in a cluster. The other members of the Cucurbitaceae, Brassicaceae and Poaceae *AOs* were more distantly related. The members of the pine and *Malus* seem to be the progenitors.

Discussion

Seabuckthorn is a real multitasked and one of nature's true super foods, seabuckthorn packs a powerful nutritional punch with a broad spectrum of health-promoting vitamins, minerals, phyto-nutrients, and essential fatty acids. Whether we use it for a specific issue or to promote overall health, one can give this little super food a try!

Vitamin C or ascorbic acid is a water-soluble vitamin most commonly found in mammals and other animals. While humans are unable to synthesize ascorbic acid and dependent on diet for their vitamin C contents. However, the chemistry of synthetic and food originated vitamin C is similar. The most fruit and vegetable are abundant in several micronutrients and photochemical contents that could affect bioavailability of vitamin C.

Ascorbate or ascorbate oxidase is the compound having multiple functions like in response to stresses, plants protection, de-toxification of ROS species, regulatory activities. It also has roles in cell growth, production of cell wall, electrons transference, like the electrons donator for various redox enzymes involved in ripening of fruits and in formation of some fruit acid (Smirnoff, 2000; Barth *et al.,* 2004; Noctor, 2006; Debolt *et al.,* 2007; Foyer & Shigeoka, 2011). Additionally, research on potatoes tuber have revealed that wounds contributed toward stimulation of ascorbate biosynthetic activity with increase in ascorbate contents (Oba *et al.,* 1994).

The isolation and sequencing of genes encoding *AO* were described in many plants. Ascorbate oxidase is an enzyme of cell wall and their mRNA coded for a principal signaling sequences distinctive of extra-cellular protein (Esaka *et al*., 1990, Ohkawa *et al*., 1989). The member of *Cucurbitaceae* family such as pumpkin, cucumber, squash, zucchini and melon are naturally occurring richest source of *AO*. The extensive biochemical and expression studies were carried out in these species (Carvalho *et al*., 1981, Esak *et al*., 1990, 1992; Lee & Dawson, 1973; Moser & Kanellis, 1994; Nakamura *et al*., 1968). Most importantly vitamin C is an antioxidant having significant role in regenerating vitamin E from oxidize forms (Carr & Frei, 1999; Bruno *et al.,* 2006). The vitamin C makes food iron contents biologically available by increasing intestinal absorptions of non hematin iron in intestine (Combs & Gerald, 2012). *AO* expressions are regulated through composite transcription and translation control (Esaka *et al*., 1992). The activities and expressions level of Ascorbate oxidase are tightly associated to cell development (Kato & Esaka, 2000).

The fruits berry is the richest source of nutrient in diet proportional to energy contents. Wild seabuckthorn berries are also good source of vitamin C. During our study we have successfully found an enlarged coding fragment of *AO* ortholog from seabuckthorn which is involved in ascorbic acid metabolism and biosynthesis. However, reliable and sensitive amplification of large cDNA fragment (2160 bp) was most important discovery achieved through RT-PCR reaction with difference of about 421 bp between length of the new and reference gene sequence. The differences were randomly distributed among the whole sequence making divergence with target gene. The differences in amplicon size were found for the gene of interest in comparison with tomato gene. Additionally to examine gene functions it is essential to clone the target gene into various types of plasmids. In case of plant biological methods the targeted gene is commonly cloned in binary-vector for the purpose of

agrobacterium transformations (Chakrabarty *et al*., 2007). The *Hr-AO* was cloned both through TA cloning and gate way cloning techniques to further study its level of expression and function.

The capability of direct cloning and selecting recombination products in Gateway cloning system is less time consuming non-laborious, and decreased possible contaminant associated with handling of transformants. In our transformation system the desired construct was firstly cloned in Entry vector (pENTR201) and was then transferred into Destination vector pXCG-mYFP (51delta 35s) SB512 by site-specific recombination. BP products (containing AO gene) were verified by sequencing. Colony PCR was used to measure the substantial Gateway LR products, because there was no change in sequence outside the recombination taking place at the Att site. A variety of recombinant pDEST vectors obtained could also be utilized for the transformation of Arabidopsis plants by floral dip system of transformation (Clough & Bent, 1998). This *Hr-AO* gene construct could potentially be used to simplify and improve the efficiencies of gene cloning in *A. tumefaciens* for transformations studies in plants or protein expression vectors and can possibly be adapted for high- throughput applications.

However extensive expression and enzyme structural studies of AO gene have been carried out but its exact function in higher plant species left unidentified. Current investigation was centered on modifications in the *Hr-AO* expression pattern in different tissues of seabuckthorn. Hence, differences in expression pattern in different tissues are quite exceptional. Gene expression changes are highly dynamic, and gene expression pattern vary and often are complex. Numerous reports showed that it is possible to improve ascorbate accumulation in plant cells via regulating the ascorbate recycling process. Ascorbate oxidase transcript accumulation, protein contents are highest in the actively growing tissues.

Gene expression in seabuckthorn tissues followed divergent profile. The increase in ascorbate level in actively growing tissues and fruits possibly will be the effect of combine actions of oxidized and recycled enzyme. It was reported already that during the process of earlier fruit growth there is an increase in transcript of *AO* showing function of the enzyme in fruit development. It has also been suggested that Ascorbate oxidase enzymes participate in regulation of cell divisions and expansions by the control of ascorbate redox reactions (Davey *et al.*, 2000; Potters *et al*., 2000; Tabata *et al*., 2001; Sanmartin *et al*., 2007). There was a massive decrease in transcript level on ripening of fruits that could partially add to higher level of reduction in ascorbic acid. In melon fruit developmental process a considerable gain in ascorbate oxidase contents during ripen of fruits have significant part in metabolic process of cell wall (Moser and Kanellis, 1994). Some results established differential regulation in expression of ascorbate oxidase gene at the levels of transcripts increase (George *et al*., 1997).

"It is just not realistic for the majority of people to devour the mandatory servings of fruits and vegetables necessitated on reliable basis, whereas taking a once-daily supplement is safe, effective, and easy to do," This combination of potentially active genes for different pathways will open a new area of understanding and is the key theme to our biofortification program.

However, diverse biosynthesis pathways were connected with specific tissue, development stage, or peripheral pressure and environmental conditions. Appreciative effort about ascorbic acid metabolisms and biosynthetic processes in fruit tissue is entailed with the purpose of regulating food plants with high ascorbic acid contents. The scrutinization of cloning and expressions of ascorbate biosynthetic enzyme included are useful approaches of this study. The assessment of cumulative data from cDNA sequence, amino acid sequence, phylogenetic reconstruction and expression pattern demonstrated that there is a considerable variability in the gene constitution.

Conclusions

The manifold roles of ascorbate oxidase in various plant physiological process and ascorbic acid biosynthesis and recycling may bequeath with a more effectual approach to improve ascorbate content of food crops. The current study exhibited isolation, cloning and sequencing of ascorbate oxidase gene from seabuckthorn. The expression studies showed that AO was vigorously expressed in dynamic fraction of the plant tissues. Identification of genes controlling ascorbic acid buildup is promising. The increasing knowledge about ascorbic acid genes should facilitate engineering AO fortified cereal crops.

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