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ORIGINAL ARTICLE

Down-regulation of *lipoxygenase* gene reduces degradation of carotenoids of golden rice during storage

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Abstract

Main conclusion **Down-regulation of lipoxygenase enzyme activity reduces degradation of carotenoids of bio-fortified rice seeds which would be an effective tool to reduce huge post-harvest and economic losses of bio-fortified rice seeds during storage.**

Bio-fortified provitamin A-enriched rice line (golden rice) expressing higher amounts of β-carotene in the rice endosperm provides vitamin A for human health. However, it is already reported that degradation of carotenoids during storage is a major problem. The gene responsible for degradation of carotenoids during storage has remained largely unexplored till now. In our previous study, it has been shown that *r9-LOX1* gene is responsible for rice seed quality deterioration. In the present study, we attempted to investigate if *r9-LOX1* gene has any role in degradation of carotenoids in rice seeds during storage. To establish our hypothesis, the endogenous lipoxygenase (LOX) activity of high-carotenoid golden indica rice seed was silenced by RNAi technology using aleurone layer and embryo-specific *Oleosin-18* promoter. To check the storage stability, LOX enzyme down-regulated high-carotenoid T_3 transgenic rice seeds were subjected to artificial aging treatment. The results obtained from biochemical assays (MDA, ROS) also indicated that after artificial aging, the deterioration of LOX-RNAi lines was considerably lower compared to β-carotene-enriched transgenic rice which had higher LOX activity in comparison to LOX-RNAi lines. Furthermore, it was also observed by HPLC analysis that down-regulation of *LOX* gene activity decreases co-oxidation of β-carotene in LOX-RNAi golden rice seeds as compared to the β-carotene-enriched transgenic rice, after artificial aging treatment. Therefore, our study substantially establishes and verifies that LOX is a key enzyme for catalyzing co-oxidation of β-carotene and has a significant role in deterioration of β-carotene levels in the carotenoid-enriched golden rice.

Keywords RNAi · β-carotene · Provitamin A · Lipoxygenase · Post-harvest deterioration · Artificial aging

Abbreviations

LOX	Lipoxygenase
MDA	Malondialdehyde
ROS	Reactive oxygen species
VAD	Vit-A deficiency
DCFDA	2′, 7′-Dichlorofluorescin diacetate

Introduction

Carotenoids are one of the most essential natural pigments belonging to the group of C_{40} isoprenoid compounds (Walter and Strack 2011; Farré et al. 2010). It is a large class of terpenoid containing widespread conjugated polyene chain which is vital for light absorption in blue–

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green range (450–550 nm) (Moise et al. 2013). It has a significant role in functioning of the visual system, growth, reduction of cancer, decrease in cardiovascular diseases, reproduction, immunity, cell differentiation and proliferation (Kim et al. 2011). However, these carotenoids are synthesized only by phototropic organisms and few non-phototropic species (Takemura et al. 2015). Hence, to meet daily requirements of carotenoids, the humans have to depend on plant sources.

The nutritional importance of carotenoids is mainly attributed to the provitamin A activity such as β-carotene, α-carotene, β-cryptoxanthin and others, with at least one intact non-oxygenated β-ionone ring (Digesù et al. 2009). Vitamin A is an important anti-nutrient and necessary nutrient for human health and its deficiency leads to night blindness, xerophthalmia, measles, etc. (Parkhi et al. 2005; Paine et al. 2005; Shumskaya and Wurtzel 2013; Guzman et al. 2010). It is estimated that over 1 billion people worldwide are suffering from malnutrition and among them, 250 million school children worldwide are suffering from Vitamin A deficiency (VAD) (Tang et al. 2009). Vitamin A can be obtained from different foods, either as preformed vitamin A in animal products (e.g., eggs and dairy products) or mainly β-carotene in plant products (e.g., dark-green leafy vegetables and fruit). However, in developing countries, VAD is the major problem owing to predominant consumption of rice as the main diet for energy source (Sellappan et al. 2009). This is because rice endosperm lacks provitamin A (β-carotene), which is converted to vitamin A in the human body. Therefore, to overcome this problem, till date, several attempts have been taken for manipulation of carotenoid biosynthetic pathway to develop β-carotene-rich golden rice (Paine et al. 2005; Ye et al. 2000; Datta et al. 2003).

Apart from increasing the carotenoid content in rice, another difficult challenge is the proper storage and maintenance of nutritional qualities of the carotenoid-enriched transgenic rice. It is well known that Lipoxygenases (LOX, linoleate: oxygen oxidoreductase, EC 1.13.11.12) are non-haem, iron-containing dioxygenases that catalyze the oxidation of polyunsaturated fatty acids (e.g., linoleic and linolenic acids) in plants, animals and microorganisms (Carrera et al. 2007). These enzymes catalyze the insertion of molecular oxygen into polyunsaturated (PUFA) lipids containing a cis, cis-1, 4-pentadiene system such as linoleic, linolenic, and arachidonic acids to yield conjugated hydroperoxide products which oxidizes carotenoids (Gardner and Grove 1998; Hidalgo and Brandolini 2008). Lipoxygenase is generally used in food-related applications in bread making, aroma production by molecular oxidation of carotenoids present in food products (Casey 1997). Conversely, the co-oxidation of carotenoids has negative effects for color, antioxidant status, off-flavor formation and nutritional quality deterioration (Baysal and Demirdöven 2007; Chedea and Jisaka 2013). The process is called "co-oxidation" because LOX does not directly act upon carotenoids, instead it produces hydroperoxy fatty acids during the catalytic reactions, which consequently co-oxidizes and decolorizes the carotenoids (Casey 1997; Wu et al. 1999). During storage, free radical produced from the intermediate steps of fatty acid oxidation such as linoleic acid is responsible for the oxidative degradation of carotenoid pigments of rice seed. The carotenoids are the most sensitive due to presence of conjugated double bond system (Wu et al. 1999) and are hence susceptible to oxidation, which poses an important storage problem and subsequent nutritional quality deterioration in β-carotene-enriched rice. It has been observed that β-carotene level of golden rice decreases rapidly during storage. Not only rice, co-oxidation of β-carotene is a most important problem for wheat too (Leenhardt et al. 2006).

In our prior study, we had identified that *r9-LOX1 gene* is responsible for seed quality deterioration. However, its (*r9-LOX1*) direct role in the degradation of carotenoid in golden rice during storage has not been studied enough. In the present study, we targeted suppression of *r9-LOX1* gene of carotenoid-enriched transgenic rice seed to find whether *r9-LOX1* gene has any role in the deterioration process. The resulting transgenic rice plants exhibited substantial diminution in LOX activity, which consequently led to reduced co-oxidation of β-carotene in LOX-RNAi golden rice seeds as compared to the β-carotene-enriched transgenic rice line (SKBR-244), after artificial aging. In addition, the LOX-RNAi golden rice lines displayed normal phenotype and no significant differences were observed in any of the agronomic traits studied when compared to wild type.

Materials and methods

Plant materials

The present experiment was performed using high-carotenoid homozygous transgenic *indica* rice (SKBR-244) developed by Datta et al. (2006). The plant was grown in green house condition to obtain seed for genetic transformation.

Cloning of RNAi vector and plant transformation

RNAi vector containing *r9-LOX1* gene under control of *Oleosin-18* promoter (*pOle-lox-006*) was used for plant transformation as described in our previous study by Gayen et al. (2014). The constructed RNAi vector *pOle-lox-006*

was introduced into the genome of golden *indica* rice line (SKBR-244), by PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA) (Datta et al. 1998). Subsequently, the transformed calli were screened on MS media containing 50 μg mL^{-1} hygromycin (Datta et al. 1990). The independent hygromycin-resistant putative transgenic plants were transferred to green house to grow subsequent generations.

Genomic DNA analysis (T_1–T_3)

The putative transgenic plants were screened by PCR analysis for the presence of transgene. Genomic DNA was extracted from leaf of putative transgenic plants according to the method described by Huang et al. (1997). The presence of transgene *psy, crtI* and RNAi vector in transgenic plant (T_1–T_3 generation) was confirmed by genomic PCR analysis using gene-specific primers. The primers sequences used were as follows: *RGA2* intron (Forward: 5′-CCTGAAATTGGTAAAAGTAGA-3 and Reverse: 5′-TGTATCTTCATACTGCATTTG-3), *psy* (Forward: 5′-TG GTGGTTGCGATATTACGA-3′ and 5′-ACCTTCCCAG TGAACACGTC-3′), *crtI* (Forward: 5′-GGTCGGGC TTATGTCTACGA-3′ and Reverse: 5′-ATACGGTCGCG AGTTTTGG-3′).

RNA isolation and qRT-PCR analysis

Expression level of different genes was verified by quantitative real-time PCR (qRT-PCR) analysis. Total RNA was extracted from matured rice seeds (30 days after pollination) using a rapid TRIzol-based method (Meng and Feldman 2010). After DNaseI treatment, RNA (4 μg) was reverse transcribed to cDNA in a 20 μl reaction volume according to manufacturer's protocol (Fermentas, USA). The resulting cDNA was used as template (1.0 μl) for PCR reaction. The quantitative PCR was performed using real-time PCR system (CFX 96 Real-time system; Bio-Rad, Hercules, CA) with SYBR green reaction mixture (Fermentas, USA) using gene-specific primers. The primers used were as follows: *r9-LOX1* (F-5′-CATCAAGGACCC ATCAAGC-3′ and R-5′-TCCCTGGAGGACACCATC-3), *Os03g0700400* (5′-GAGATCGAAGGGAAGGTGGT-3′ and 5′-CGGAGGTGTTGGGGTAAAG-3′), *LOX-L2* (5′- A CGCTGCTCTACCCAAACAC-3′ and 5′-CCGGTAAAG ACCACCGTAAA-3′), *RLL* (5′-GAGGCGATCAACCAG AAGAG-3′ and 5′-GTCGTCGGTGAGGAAGAAGA-3′), *crtI* (5′-CGATCCCAGTGCCATTGAAG-3′ and 5′-CA TCCCCTGAACTAATGCGC-3′) and *psy* (5′-ATGCTTA TGATCGATGCGGC-3′ and 5′-CCTCTTCTTGCATCTT CGCC-3′). For each reaction, *β-tubulin* (BTF-5′-GGAG TCACATGCTGCCTAAGGTT-3 and BTR-5′-TCACTG CCAGCTTACGGAGG-3′) was used as reference gene. The quantitative variation between samples was calculated by ΔΔCt method.

Southern blot analysis

Southern blot analysis was performed for confirmation of stable integration. The transgene integration, stability and trait improvement in rice have been discussed by Datta et al. (1997). 10 μg of genomic DNA was digested with respective restriction enzyme at 37 °C for overnight and total volume of digestion was 150 μl. The digested genomic DNA was electrophoresed at 50 V in 1 % agarose. The DNA fragments were transferred to Hybond-N^+ membrane using 10 × SSC solutions for overnight according to manufacturer's protocol. The blot was hybridized with *crtI* labeled with α^{32}P dCTP isotope (BARC, India) and *RGA2* intron labeled with DIG using Random Primed DNA labeling kit (Roche, USA).

Enzyme extraction and lipoxygenase enzyme assay

Total protein from rice seeds was extracted by 0.1 M sodium phosphate buffer (pH-6.3) and LOX activity was measured by spectrophotometer (Bio-Rad, USA) at A_{234} at 25 °C as described by Mizuno et al. (2003). LOX activity of rice seeds was expressed as Δ_{234}/min/mg of protein.

Artificial aging treatment of transgenic and non-transgenic rice seeds

Artificial aging of matured seeds was performed in a plant growth chamber (Eyela, Japan) at 45 ± 1 °C and 85 % relative humidity in dark condition for 14 days (Li et al. 2007).

Lipid peroxidation assay

Lipid peroxidation was measured by MDA contents of mature rice seeds (transgenic LOX down-regulated line and β-carotene-enriched rice seed), as described by Gayen et al. (2014). The MDA content was measured by extinction coefficient 155 mM^{-1} cm^{-1} and expressed as nmole/g.

Estimation of total ROS by DCFDA

100 mg of rice seed powder was taken in 2.0 ml microcentrifuge tube and vortex with 1.0 ml 10 mM Tris–HCl pH-7.2. Extract was collected after centrifugation at 12,000 rpm for 20 min. Fluorescence intensity was measured with 1 mM DCFDA at Ex-495 nm and Em-529 nm

(Jambhunathan 2010). The ROS value was expressed by relative intensity per mg of protein.

Carotenoids extraction and HPLC analysis

Carotenoids were extracted from powder mature rice seed (100 mg) using 1 ml of NaCl (200 g/L) and 1 ml of 1:1 cyclohexane and ethyl acetate solvent. Prior to extraction, 20 µl of Apocarotenal was added (10 µg/ml) to the extraction mixture as an internal standard. The mixture was vortexed and centrifuged at 2000 rpm for 10 min. The upper phase was collected and the extraction process was repeated until the elimination of yellow color from rice sample. The extract was dried in speedvac system (Eyela, Japan) and dissolved in 50 µl 1:1 cyclohexane and ethyl acetate mixture. Carotenoids were separated with YMC C30 column using methanol, tertiary butyl methyl ether and water mixture (81:15:4–6:90: 4) (Sander et al. 1994).

Statistical analysis

All the data were analyzed by Graphpad prism 5 software and expressed as mean ± SE (standard error). The mean values were compared by ANOVA and statistical significant differences were analyzed using Bonferroni Post-tests.

Results

Generation of *LOX-RNAi* transgenic golden rice lines

Transgenic golden rice containing high level of carotenoids was developed by integration of phytoene synthase (*psy*) and phytoene desaturase (*crt*I) into a high-yielding rice cultivar BR29 (Fig. 1a) (Datta et al. 2006). In our previous studies, it was reported that lipoxygenase present in rice seed is responsible for nutritional quality deterioration and viability (Gayen et al. 2014). Therefore, in order to suppress co-oxidation of β-carotene of golden rice, LOX activity of the transgenic rice seeds was reduced by silencing of *r9-LOX1* (AB099850) gene by Gateway-based RNAi technology using tissue-specific *Oleosin-18* promoter (Gayen et al. 2014). For down-regulation of LOX activity, partial fragment of *r9-LOX1* gene was cloned in sense and antisense orientation under the control of *Oleosin-18* promoter (Fig. 1b). The tissue-specific *Oleosin-18* promoter was used to reduce LOX activity specifically in the embryo and aleurone layer of rice grains (where LOX is generally present). Through Biolistic transformation and subsequent tissue culture, twenty-four putative hygromycin-resistant transgenic plants were obtained. Genomic DNA was isolated from all twenty-four independent plants along with control plant (Golden rice without RNAi-LOX construct) and subjected to PCR analysis using specific primer pairs for amplification of wheat *RGA2* intron to confirm the integration of *pOle-lox-006* vector in the plant genome. Simultaneously, presence of *psy* and *crtI* gene of golden rice (T_0–T_3) was checked by PCR analysis using gene-specific primers (Rai et al. 2007). Out of 24 plants, 20 plants showed amplified fragments of *RGA2* intron (310 bp), *crtI* and *psy,* respectively. There were no corresponding bands in the negative control and wild-type plants. Subsequently, down-regulation of LOX activity in these PCR positive transgenic plants (T_0) was confirmed by LOX enzyme assay. Among the transgenic lines analyzed, three T_0 transgenic lines exhibiting maximum down-regulation of LOX activity with respect to β-carotene-enriched transgenic line SKBR-244 were selected and successive generations were grown under greenhouse condition until maturity (T_1–T_3). Segregation of transgenic plants (T_1–T_3) was analyzed by genomic PCR analysis. Down-regulation of LOX activity of transgenic T_2 plants was confirmed by LOX enzyme assay. After screening of resultant transgenic generations (T_1–T_3), most promising LOX down-regulated T_3 transgenic lines #10-4-2, #14-10-4 and #15-5-7 were selected for further molecular and biochemical analyses. Integration of *crtI*, psy and *lox* gene was confirmed by genomic PCR analysis (Fig. 1c).

Reduced LOX activity

The reduction of LOX activity in LOX-RNAi transgenic golden rice seeds (T_3) was measured by enzyme assay using linoleic acid as substrate. The enzyme assay revealed that maximum down-regulation was observed in #10-4-2 (69.0 %), #14-10-4 (67.0 %) and #15-5-7 (64 %) lines compared to that of β-carotene-enriched transgenic rice seed, indicating a seed-specific down-regulation by tissue-specific *Oleosin-18* promoter (Fig. 1d).

Expression analysis of transgenic T_3 plant

The mRNA expression level of *lox* gene in different transgenic lines (T_3) was analyzed by qPCR analysis using total RNA extracted from rice seeds. The qPCR analysis revealed that the transcript level of *r9-LOX1*gene (AB099850) was reduced significantly in #10-4-2 (68.0 %), #14-10-4 (67.0 %) and #15-5-7 (62.0 %) transgenic T_3 lines with respect to β-carotene-enriched SKBR-244 rice. In addition, the transcript level of *Os03g0700400* gene having sequence similarity with *r9-LOX1* gene was also declined by 59 % in #10-4-2, 58.0 % in #14-10-4 and 48 % in #15-5-7 lines as compared to β-carotene-enriched transgenic rice (Fig. 2) (Gayen et al. 2014). Expression

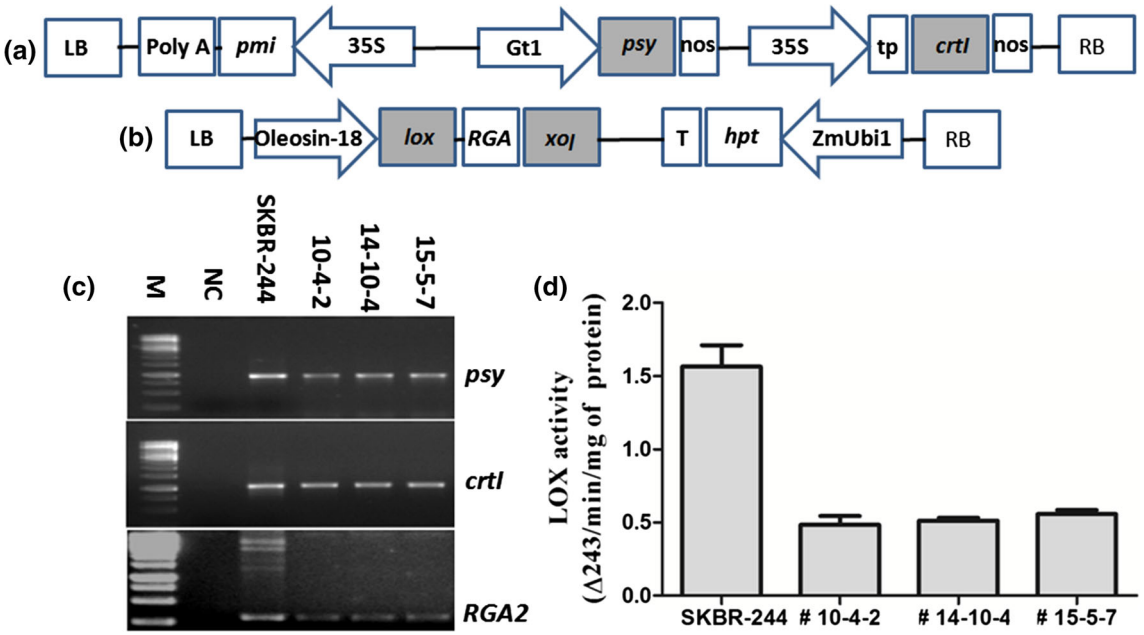

Fig. 1 Schematic diagram of RNAi vector used for rice transformation and analysis of transgenic plant (T_3) # 10-4-2, # 14-10-4 and # 15-5-7 by genomic PCR and RT-PCR analysis. **a** Binary vector containing *psy* and *crtI* gene was used for plant transformation. **b** Binary vector construct containing partial fragment of *r9-LOX1* gene under control of tissue-specific *Oleosin-18* promoter. **c** PCR confirmation of *psy*, *crtI* and *RGA2* gene as amplified from genomic DNA of transgenic rice plant. **d** Lipoxygenase enzyme assay of transgenic rice seeds (T_3) # 10-4-2, # 14-10-4 and # 15-5-7 with respect to SKBR-244 rice seeds. Values presented as mean ± SE ($n = 3$)

level of LOX-L2 (X64396) and RLL (D14000) was also checked to verify if any alteration due to silencing of LOX gene. The results clearly indicated that there was no alteration in gene expression due to silencing of desired gene (LOX) to enhance storage stability of carotenoid-enriched golden rice. Furthermore, the expression of both *psy* and *crt*I genes was confirmed by semi-quantitative RT-PCR analysis. The results confirmed expression of both *psy* and *crt*I in transgenic LOX-RNAi golden rice seed (Fig. 3).

Southern hybridization

The stable integration of trans gene in the genome of transgenic lines #10-4-2, #14-10-4, #15-5-7 was confirmed by Southern blot analysis using gene-specific probe *psy* and *RGA2*. The isolated genomic DNA was digested with *EcoRI* and *SacI* restriction enzyme. The results revealed the stable integration of the transgene into the progeny (T_3). The Southern hybridization showed band in transgenic plants #10-4-2, #14-10-4, #15-5-7. However, no hybridization band was detected in case of the non-transgenic control plants (Fig. 4).

Down-regulation of LOX enhances storage stability

Both transgenic and SKBR-244 rice seeds were subjected to artificial aging treatment at 45 °C and 85 % relative humidity (RH) for 14 days. Prior to aging treatment, LOX activity of LOX-RNAi transgenic lines #10-4-2, #14-10-4, #15-5-7 and SKBR-244 rice seeds was 0.48, 0.51, 0.55 and 1.56 Δ234/min/mg of protein. However, the results obtained after aging clearly showed higher LOX activity in SKBR-244 seeds (2.40 Δ234/min/mg of protein) as compared to that of transgenic #10-4-2 (0.64 Δ234/min/mg of protein), #14-10-4 (0.65 Δ234/min/mg of protein) and #15-5-7 (0.69 Δ234/min/mg of protein), respectively (Fig. 5a).

Subsequently, lipid peroxidation of rice seeds was also determined by MDA assay. From this study, it was observed that initially MDA content of SKBR-244 rice seed and transgenic lines #10-4-2, #14-10-4, #15-5-7 was 5.2, 3.71, 3.73 and 3.90 nmol/g, respectively. However, after aging treatment, MDA content of SKRB-244 rice increased more rapidly (6.56 nmol/g) compared to *lox* down-regulated transgenic rice lines #10-4-2 (4.3 nmol/g), #14-10-4 (4.33 nmol/g) and #15-5-7 (4.83 nmol/g), respectively (Fig. 5b). Simultaneously, we measured ROS content in rice seeds by fluorometric assay. The results displayed a significant increase in the ROS content of SKBR-244 rice seeds from 8.19 RFU/μg of protein to 12.66 RFU/μg of protein whereas the level of ROS content increased marginally in transgenic carotenoid lines #10-4-2 (8.01–8.19 RFU/μg of protein), #14-10-4 (8.0–8.52 RFU/μg of protein) and #15-5-7 (8.13–8.78 RFU/μg of protein) (Fig. 5c).

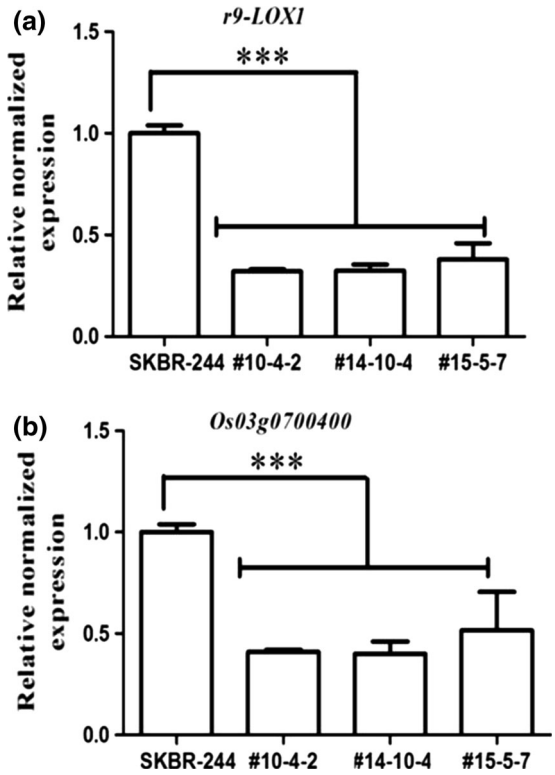

Fig. 2 Expression level analysis of **a** *r9-LOX1*. **b** *Os03g007400* gene of transgenic rice seeds (T_3) # 10-4-2, # 14-10-4 and # 15-5-7 by quantitative real-time PCR with respect to β-carotene-enriched transgenic line SKBR-244 using SYBR green. All the values were normalized using *β-tubulin* as reference gene. Values presented as mean ± SE ($n = 3$)

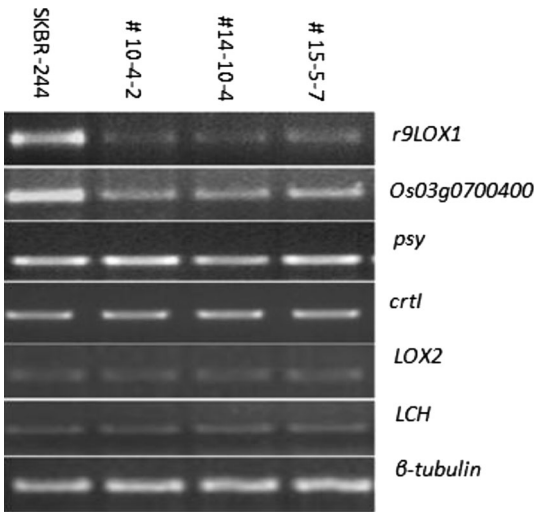

Fig. 3 RT-PCR analysis of different gene of LOX down-regulated transgenic (# 10-4-2, # 14-10-4 and # 15-5-7) rice seeds as compared to control. *β-tubulin* was used as reference gene

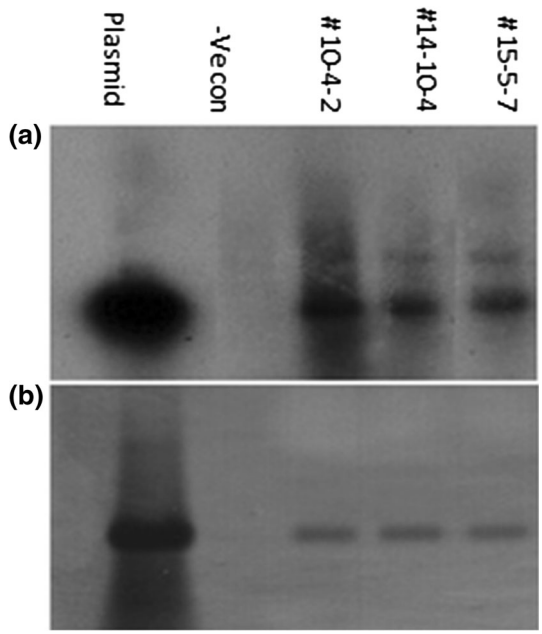

Fig. 4 Southern blot analysis of transgenic plant (T_3) # 10-4-2, # 14-10-4 and # 15-5-7 with respect to SKBR-244 plant. **a** 10 μg genomic DNA was digested with *EcoR*1 enzyme and hybridized with *crtI* probe (radioactive). **b** 10 μg genomic DNA was digested with *Sac*I enzyme and hybridized with *RGA2* probe (non-radioactive)

Down-regulation of LOX reduces deterioration of carotenoids during storage

To determine whether down-regulation of LOX activity actually enhances storage stability of rice seeds, carotenoid content (β-carotene, α-carotene and lutein) of rice seeds was analyzed by HPLC method after artificial storage (Fig. 6). Data obtained from HPLC analysis evidently illustrated that before artificial aging treatment β-carotene of LOX-RNAi transgenic lines #10-4-2, #14-10-4, #15-5-7 and SKBR-244 rice was 3.21, 3.18, 3.18 and 3.30 μg/g, respectively. Whereas, after aging treatment, β-carotene content of SKBR-244 rice seed (56.0 %) decreased significantly as compared to that of transgenic LOX down-regulated lines #10-4-2 (9.6 %), #14-10-4 (11.3 %), #15-5-7 (24.7 %). Similar trends were observed in case of α-carotene and lutein. This result clearly demonstrates that the amount of LOX levels in the rice seeds is directly involved in degradation of carotenoid (Fig. 7).

Agronomic evaluation of transgenic LOX-RNAi plant

A phenotypic evaluation of transgenic LOX-RNAi and β-carotene-enriched SKBR-244 plants revealed no major

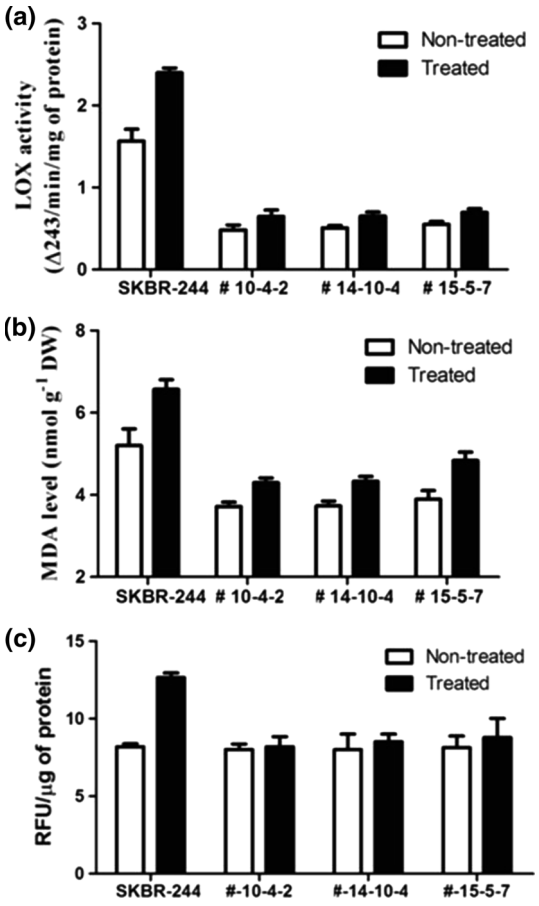

Fig. 5 Analysis of LOX attenuated transgenic rice seeds # 10-4-2, # 14-10-4 and # 15-5-7 with respect to SKBR-244 after accelerated aging treatment at 45 °C and relative humidity at 85 % for 14 days. **a** LOX enzyme assay of rice seed. **b** MDA production of rice seed. **c** Estimation of ROS formation by H_2CDFDA fluorescence indicator. Values presented as mean ± SE ($n = 3$)

differences at the vegetative growth stage. No significant alterations were observed in plant height, seeds weight, panicle length, number of panicle, etc., of transgenic and SKBR-244 plants grown under greenhouse condition. From this study, it was revealed that the down-regulation of *lox* gene did not affect any of the morphological traits observed in transgenic LOX-RNAi and, therefore, the SKBR-244 and three lox down-regulated transgenic plants were phenotypically similar (Table 1).

Discussion

The carotenoids are nonpolar compounds, having diverse roles in human nutrition and serve numerous biological functions (Digesù et al. 2009). Owing to their antioxidant properties, carotenoids have been generally associated with reduced incidence of cancers, cardiovascular diseases, macular degeneration, cataracts and other photosensitive conditions (Digesù et al. 2009; García-Casal 2006). Among different carotenoids, β-carotene is often referred to as provitamin A, as it can be cleaved into vitamin A within human body and is, therefore, attributed to be an important carotenoid for human nutrition (Paine et al. 2005). Till date, numerous approaches have been reported for increment of β-carotene levels in rice (Paine et al. 2005; Ye et al. 2000; Datta et al. 2003, 2006). However, these Provitamin A carotenoids including β-carotene in the staple food crops are not stable during storage and thus lead to quality deterioration (Li et al. 2007). The post-harvest losses are even worse in developing countries like India, due to storage of crops in higher temperature and humid environment. Prior reports suggest that β-carotene is considerably more susceptible to oxidation due to presence of conjugated double bond system (Leenhardt et al. 2006). In our previous study, we established that *r9-LOX1* gene of rice seeds was responsible for seed quality deterioration during storage (Gayen et al. 2014). In the present study, we have developed transgenic golden rice with reduced LOX activity by silencing endogenous *lipoxygenase* gene to reduce co-oxidation of carotenoids using RNAi technology which is very powerful tool for crop improvement (Saurabh et al. 2014). The RNAi-mediated gene silencing of *lox* gene was driven by tissue-specific *Oleosin-18* promoter instead of any constitutive promoter to avoid any undesirable and detrimental effects on the plant (Kuwano et al. 2009; Ali et al. 2013).

On the basis of qPCR analysis and simultaneous LOX assay, the most promising *lox* down-regulated transgenic lines #10-4-2, #14-10-4 and #15-5-7 were selected. The T_3 transgenic seeds showed pronounced silencing of both *r9-LOX1* and *Os03g0700400* due to sequence similarity as reported in our earlier studies (Gayen et al. 2014). However, no change was observed in the transcript level of other related lipoxygenase genes such as *LOX-L2* and *RLL* which were also analyzed by RT-PCR to check if there was any alteration in their gene expression with respect to β-carotene-enriched transgenic SKBR-244 rice seeds. The expression of *psy* and *crtI* gene in both SKBR-244 and transgenic LOX-RNAi seeds was analyzed by semi-quantitative RT-PCR as shown (Datta et al. 2003). Stable integration of lipoxygenase gene was confirmed by Southern blot analysis.

To substantiate the storage stability of carotenoids in the transgenic LOX-RNAi seeds, both transgenic and β-carotene-enriched transgenic SKBR-244 rice seeds were aged artificially at 45 °C and 85 % RH for 14 days as reported in earlier reports (Li et al. 2007; Zhang et al. 2007). In general, LOX activity is increased due to storage (artificial aging treatment) (Suzuki et al. 1999), which is evident from the increment of LOX activity in SKBR golden rice seeds as compared to LOX-RNAi seeds, which showed

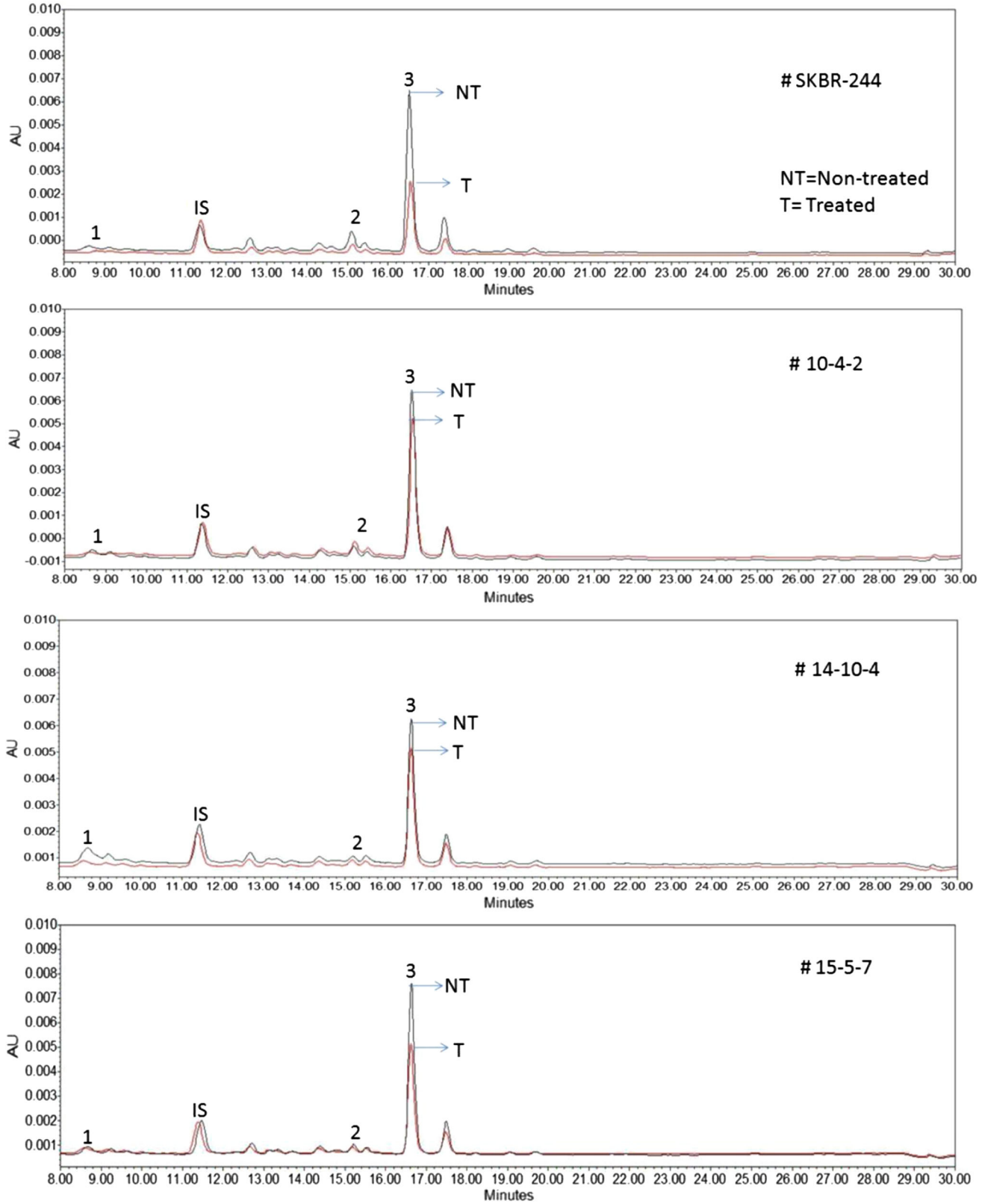

Fig. 6 HPLC analysis of carotenoids of transgenic rice seeds # 10-4-2, # 14-10-4 and # 15-5-7 with respect to β-carotene-enriched transgenic line SKBR-244 after accelerated aging treatment at 45 °C and relative humidity at 85 % for 14 days. Values presented as mean ± SE ($n = 3$). *1* Lutein, *2* α-carotene, *3* β-carotene and *IS* internal standard. *Red line* indicates aging treated and *black line* represents for non-treated seed

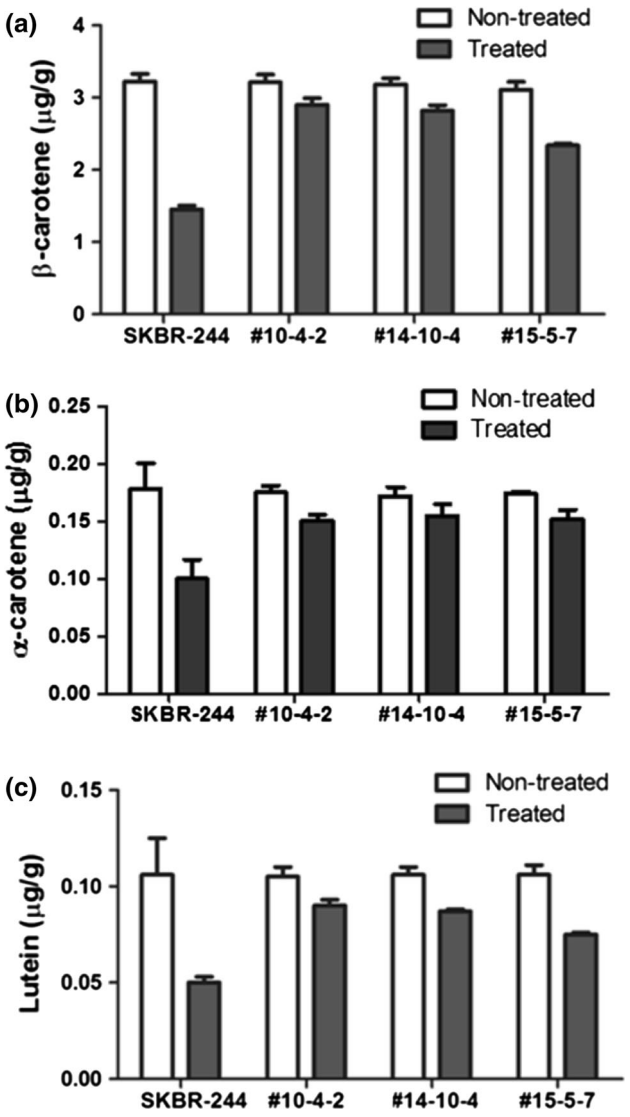

Fig. 7 Estimation of carotenoids content of transgenic rice seeds # 10-4-2, # 14-10-4 and # 15-5-7 with respect to β-carotene-enriched transgenic line SKBR-244 after aging treatment

minimal increase in the activity after aging treatment. It has also been reported that ROS and MDA are produced by fatty acid oxidation due to the action of LOX enzyme, which has detrimental effects on seed viability and nutritional quality deterioration (Gayen et al. 2014; Repetto et al. 2012). Therefore, storage stability of artificially aged transgenic rice seeds was evaluated by MDA and ROS assay. It was observed that SKBR-244 rice seeds showed significant increase in MDA content as compared to the transgenic lox down-regulated lines. ROS produced as a result of fatty acid oxidation leads to tissue damage and degradation of nutritional components including seed carotenoids, mainly β-carotene (Sharma et al. 2012). We detected ROS in rice seeds (SKBR-244 and LOX-RNAi) using fluorescent probe DCFDA and observed that LOX-RNAi seeds formed lower amount of ROS when compared to SKBR-244 rice seeds during storage. This minimal formation of ROS in LOX-RNAi seeds owing to efficient down-regulation of *lox* was comparable to the findings reported earlier (Gayen et al. 2014). Consequently, all these results clearly suggest that lipid oxidation of transgenic LOX-RNAi golden rice seeds was minimum compared to that of SKBR-244 rice seeds and, therefore, storage stability of lox down-regulated transgenic rice was enhanced considerably with respect to SKBR-244.

Co-oxidation of β-carotene has been a serious problem for maize, wheat, cassava, etc. It was reported that commercial high-carotenoid yellow maize lines lost about 30–56 % of provitamin A content during long-term storage (Quackenbush 1963; Dua et al. 1965). In case of yellow wheat, approximately 20–48 % total provitamin A was lost when stored even at 20 °C for 20 days. All these reports evidently suggest that β-carotene deterioration during storage is a serious problem and, therefore, in this study our major objective was to stabilize the carotenoid content of β-carotene-enriched golden rice from co-oxidation caused by LOX enzyme during storage. This was validated by HPLC analysis that transgenic LOX-RNAi seeds which exhibited minimal deterioration of carotenoids when compared to SKBR-244 golden rice seeds after artificial aging. These observations evidently substantiates that efficient silencing of LOX activity by RNAi technology enhanced the storage stability of carotenoid-enriched golden rice which will reduce Vitamin A deficiency (Datta 2004).

Agronomic evaluation is very important aspect of transgenic research and, therefore, we compared different agronomic traits of the resultant transgenic LOX-RNAi rice plants with respect to SKBR-244 plants. All the morphological characters studied in SKBR-244 and transgenic plants were comparable and suggest that all transgenic LOX-RNAi plants were phenotypically normal and fertile.

Table 1 Phenotypic evaluation of transgenic (T_3) rice plant with respect to high-carotenoid golden rice SKBR-244

Rice plant	Plant height (cm)	Panicle length (cm)	No of tiller	1000 seeds weight (gm)
# SKBR-244	83.33 ± 1.74	22.66 ± 0.60	16.35 ± 0.33	22.36 ± 0.20
# 10-4-2	83.66 ± 1.20	22.33 ± 0.33	16.29 ± 0.57	22.07 ± 0.25
#14-10-4	83.66 ± 1.85	22.20 ± 0.48	16.23 ± 0.88	22.31 ± 0.46
# 15-5-7	83.16 ± 1.64	22.03 ± 0.26	16.40 ± 0.64	22.70 ± 0.11

From this study, it is confirmed that *r9-LOX1* is responsible for degradation of carotenoids of bio-fortified golden rice. Therefore, reduction of LOX activity by RNAi-mediated silencing would be an effective tool to reduce huge post-harvest and economic losses during storage process.

Author contribution KD, SKD and DG conceived and designed the research. DG, NA and SNS conducted experiment and analyzed data. DG wrote the paper. KD, SKD, NA and SNS revised the manuscript critically and approved for publication.

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References

Ali N, Paul S, Gayen D, Sarkar SN, Datta K, Datta SK (2013) Development of low phytate rice by RNAi mediated seed-specific silencing of inositol 1,3,4,5,6 pentakisphosphate 2-Kinase gene (IPK1). PLoS One 8:1–12

Baysal T, Demirdöven A (2007) Lipoxygenase in fruits and vegetables: a review. Enzyme Microb Tech 40:491–496

Carrera A, Echenique V, Zhang W, Helguera M, Manthey F, Schrager A, Picca A, Cervigni G, Dubcovsky J (2007) A deletion at the Lpx-B1 locus is associated with low lipoxygenase activity and improved pasta color in durum wheat (*Triticum turgidum ssp. durum*). J Cereal Sci 45:67–77

Casey R (1997) Lipoxygenases and breadmaking. In: Angelino SAGF, Hamer RJ, van Hartingsfeld W, Heidekamp F, van der Lugt JP (eds). In: Proceedings of the first European symposium on enzymes and grain processing, pp 188–194

Chedea VS, Jisaka M (2013) Lipoxygenase and carotenoids: a co-oxidation story. Afr J Biotechnol 12:2786–2791

Datta SK (2004) Rice biotechnology: a need for developing countries. Agbioforum 7:31–35

Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile indica-rice recovered from protoplasts. Nat Biotechnol 8:736–740

Datta SK, Torrizo LB, Tu J, Oliva NP, Datta K (1997) Production and molecular evaluation of transgenic rice plants. In: IRRI discussion paper series no. 21. International Rice Research Institute, Manila, pp 35–39

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998) Constitutive and tissue-specific differential expression of the *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest. Theor Appl Genet 97:20–30

Datta K, Baisakh N, Oliva N, Torrizo L, Abrigo E, Tan J, Rai M, Rehana S, Al-Babili S, Bayer SP, Potrykus I, Datta SK (2003) Bioengineered 'golden' indica rice cultivars with beta-carotene metabolism in the endosperm with hygromycin and mannose selection systems. Plant Biotechnol J 1:81–90

Datta K, Rai M, Parkhi V, Oliva N, Tan J, Datta SK (2006) Improved 'golden' indica rice and post-transgeneration enhancement of metabolic target products of carotenoids (β-carotene) in transgenic elite cultivars (IR64 and BR29). Curr Sci 91:935–939

Digesù AM, Platani C, Cattivelli L, Mangini G, Blanco A (2009) Genetic variability in yellow pigment components in cultivated and wild tetraploid wheats. J Cereal Sci 50:210–218

Dua PN, Day EJ, Grogan CO (1965) Loss of carotenoids in stored commercial and high-carotenoid yellow Corn, *Zea mays* L. Agron J 57:501–502

Farré G, Sanahuja G, Naqvi S, Bai C, Capell T, Zhu C, Christou P (2010) Travel advice on the road to carotenoids in plants. Plant Sci 179:28–48

Garcıa-Casal MN (2006) Carotenoids increase iron absorption from cereal-based food in the human. Nutr Res 26:340–344

Gardner HW, Grove MJ (1998) Soybean Lipoxygenase-1 Oxidizes 3*Z*-Nonenal. Plant Physiol 116:1359–1366

Gayen D, Ali N, Ganguly M, Paul S, Datta K, Datta SK (2014) RNAi mediated silencing of *lipoxygenase* gene to maintain rice grain nutritional quality and viability during storage. Plant Cell Tissue Organ Cult 118:229–243

Guzman I, Hamby S, Romero J, Bosland PW, O'Connell MA (2010) Variability of carotenoid biosynthesis in orange colored *Capsicum* spp. Plant Sci 179:49–59

Hidalgo A, Brandolini A (2008) Kinetics of Carotenoids Degradation during the Storage of Einkorn (*Triticum monococcum* L. ssp. *monococcum*) and Bread Wheat (*Triticum aestivum* L. ssp. *aestivum*) Flours. J Agri Food Chem 56:11300–11305

Huang N, Angeles ER, Domingo J, Magpantay G, Singh S, Zhang G, Kumaravadivel N, Bennett J, Khush GS (1997) Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theor Appl Genet 95:313–320

Jambhunathan N (2010) Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. Methods Mol Biol 639:292–298

Kim D, Han S, Song J (2011) Evaluation of the inorganic compound leakage and carbohydrates as indicator of physiological potential of *Ulmus parvifolia* seeds. New Forest 41:3–11

Kuwano M, Mimura T, Takaiwa F, Yoshida KT (2009) Generation of stable 'low phytic acid' transgenic rice through antisense repression of the 1 D -myoinositol–phosphate synthase gene using the 18-kDa oleosin promoter. Plant Biotechnol J 7:96–105

Leenhardt F, Lyan B, Rock E, Boussard A, Potus J, Chanliaud E, Remesy C (2006) Wheat lipoxygenase activity induces greater loss of carotenoids than vitamin E during breadmaking. J Agri Food Chem 54:1710–1715

Li J, Zhang Y, Yu Z, Wang Y, Yang Y, Liu Z, Jiang J, Song M, Wu Y (2007) Superior storage stability in low lipoxygenase maize varieties. J Stored Prod Res 43:530–534

Meng L, Feldman L (2010) A rapid TRIzol-based two-step method for DNA-free RNA extraction from Arabidopsis siliques and dryseeds. Biotechnol J 5:183–186

Mizuno K, Iida T, Takano A, Yokoyama M, Fujimura T (2003) A new *9-lipoxygenase* cDNA from developing rice seeds. Plant Cell Physiol 44:1168–1175

Moise AR, Al-Babili S, Wurtzel ET (2013) Mechanistic aspects of carotenoid biosynthesis. Chem Rev 114:164–193

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Hinchliffe SY, Adams E, Silverstone JL, Drake ALR (2005) Improving the nutritional value of golden rice through increased pro-vitamin A content. Nat Biotechnol 23:482–487

Parkhi V, Rai V, Tan J, Oliva N, Rehana S, Bandopadhyay A, Torrizo L, Ghole V, Datta K, Datta SK (2005) Molecular characterization of marker-free transgenic lines of indica rice that accumulate β-carotene in the endosperm. Mol Genet Genom 274:325–336

Quackenbush FW (1963) Corn carotenoids: effects of temperature and moisture on losses during storage. Cereal Chem 40:266–269

Rai M, Datta K, Parkhi V, Tan J, Oliva N, Chawla HS, Datta SK (2007) Variable T-DNA linkage configuration affects inheritance

of carotenogenic transgenes and carotenoid accumulation in transgenic indica rice. Plant Cell Rep 26:1221–1231

Repetto M, Semprine J, Boveris A (2012) Lipid Peroxidation: Chemical Mechanism, Biological Implications and Analytical Determination, Lipid Peroxidation, Dr. Angel Catala (Ed.). ISBN: 978-953-51-0716-3

Sander LC, Sharpless KE, Craft NE, Wlse SA (1994) Development of engineered stationary phases for the separation of carotenoid isomers. Anal Chem 66:1667–1674

Saurabh S, Vidyarthi AS, Prasad D (2014) RNA interference: concept to reality in crop improvement. Planta 239:543–564

Sellappan K, Datta K, Parkhi V, Datta SK (2009) Rice caryopsis structure in relation to distribution of micronutrients (iron, zinc, b-carotene) of rice cultivars including transgenic indica rice. Plant Sci 177:557–562

Sharma P, Jha AB, Dubey RS, Pessarakl M (2012) Reactive oxygen species, oxidative damage, and antioxidative defensemechanism in plants under stressful conditions. J Bot 2012:1–26

Shumskaya M, Wurtzel ET (2013) The carotenoid biosynthetic pathway: thinking in all dimensions. Plant Sci 208:58–63

Suzuki Y, Ise K, Li C, Honda I, Iwai Y, Matsukura U (1999) Volatile components in stored rice [*Oryza sativa* (L.)] of varieties with and without lipoxygenase-3 in seeds. J Agric Food Chem 47:1119–1124

Takemura M, Maoka T, Misawa N (2015) Biosynthetic routes of hydroxylated carotenoids (xanthophylls) in *Marchantia polymorpha*, and production of novel and rare xanthophylls through pathway engineering in *Escherichia coli*. Planta 241:699–710

Tang G, Qin J, Dolnikowski GG, Russell RM, Grusak MA (2009) Golden rice is an effective source of vitamin A. Am J Clin Nutr 89:1776–1783

Walter MH, Strack D (2011) Carotenoids and their cleavage products: biosynthesis and functions. Nat Prod Rep 28:663–692

Wu Z, Robinson DS, Hughes RK, Casey R, Hardy D, West SI (1999) Co-oxidation of beta-carotene catalyzed by soybean and recombinant pea lipoxygenases. J Agric Food Chem 47:4899–4906

Ye X, Al-Babili S, Klöti A, Zhang AJ, Lucca P, Bayer P, Potrykus I (2000) Engineering the provitamin A (β-Carotene) biosynthetic pathway into (Carotenoid-Free) rice endosperm. Science 287:303–305

Zhang Y, Yu Z, Lu Y, Wang Y, She D, Song M, Wu J (2007) Effect of the absence of lipoxygenase isoenzymes on the storage characteristics of rice grains. J Stored Prod Res 43:87–91