Gene 649 (2018) 40–49

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Characterization of metabolic network of oxalic acid biosynthesis through RNA seq data analysis of developing spikes of finger millet (*Eleusine coracana*): Deciphering the role of key genes involved in oxalate formation in relation to grain calcium accumulation

Naved Akbar[a,b], Supriya Gupta[a], Apoorv Tiwari[a], K.P. Singh[b], Anil Kumar[a,*]

[a] Department of Molecular Biology & Genetic Engineering, College of Basic Sciences & Humanities, G B Pant University of Agriculture & Technology, Pantnagar 263145, Uttarakhand, India
[b] Department of Biotechnology, Shri Venkateshwara University, Gajraula 244235, Uttar Pradesh, India

ARTICLE INFO

Keywords:
Calcium
Oxalic acid
Tartaric acid
Antinutrient
Finger millet
Transcriptome

ABSTRACT

In the present study, we identified seven major genes of oxalic acid biosynthesis pathway (SGAT, GGAT, ICL, GLO, MHAR, APO and OXO) from developing spike transcriptome of finger millet using rice as a reference. Sequence alignment of identified genes showed high similarity with their respective homolog in rice except for OXO and GLO. Transcript abundance (FPKM) reflects the higher accumulation of identified genes in GP-1 (low calcium genotype) as compared to GP-45 (high calcium genotype) which was further confirmed by qRT-PCR analysis, indicating differential oxalate formation in both genotypes. Determination of oxalic acid and tartaric acid content in developing spikes explain that higher oxalic acid content in GP-1 however, tartaric acid content was more in GP-45. Higher calcium content in GP-45 and lower oxalate accumulation may be due to the diversion of more ascorbic acid into tartaric acid and may correspond to less formation of calcium oxalate. Our results suggest that more than one pathway for oxalic acid biosynthesis might be present in finger millet with probable predominance of ascorbate-tartarate pathway rather than glyoxalate-oxalate conversion. Thus, finger millet can be use as an excellent model system for understanding more specific role of nutrients-antinutrients interactions, as evident from the present study.

1. Introduction

Finger millet (*Eleusine coracana*) is an orphan crop belonging to the poaceae family. In India, after pearl millet (*Pennisetum glaucum*), finger millet is a third important staple millet and in the world its rank fourth in importance among other millets after sorghum, pearl millet and foxtail millet (Sood et al., 2017). It is highly nutritious as its grains contain 65–75% carbohydrates, 5–8% protein, 15–20% dietary fiber and 2.5–3.5% minerals (Kumar et al., 2016) and an excellent source of calcium (up to 450 mg/100 g seed) which is far above than other millets and cereals (Panwar et al., 2010). Ca^{+2} is one of the essential mineral nutrients for humans because it has absolutely necessary structural and signaling roles (Ross et al., 2011). Low dietary calcium intake has been associated with diseases such as rickets (Bhatia, 2008) and osteoporosis (Chan et al., 2006); both are conditions of low bone fragility in humans.

Being rich in grain calcium, finger millet can be a good model crop to study the molecular mechanisms of calcium accumulation in relation to other sequestering molecules. Calcium present in plant foods exists primarily as a complex in which it is bound to substances such as phytate, oxalate, fatty acid, protein, fiber and other anions (Wilson and Stephenson, 1990; Linder, 1991). Several nutritional studies suggest that oxalate is an antinutrient that makes calcium unavailable for nutritional digestion by human (Heaney and Weaver, 1989; Weaver et al., 1987) and absorption of calcium appear to be inversely proportional to the oxalate content in food (Weaver et al., 1987). The role of calcium sensor and transport gene family in seed calcium accumulation in developing spikes of finger millet genotypes differing in grain calcium was investigated earlier in our lab (Singh et al., 2014a, 2015). However, it is equally important to elucidate the role of sequestering molecules such as oxalate in grain calcium accumulation in finger millet.

Abbreviations: SGAT, Serine Glyoxalate Amino-Transferase; GGAT, Glutamate Glyoxylate Amino-Transferase; ICL, Isocitrate Lyase; GLO, Glyoxal Oxidase; MHAR, Mono Dehydro Ascorbate Reductase; APO, Ascorbate Per Oxidase; OXO, Oxalate Oxidase; FPKM, Fragments Per Kilobase of exon per Million fragments mapped; RMSD, Root Mean Square Deviation; GP-1, GPHCPB-1 (low calcium genotype); GP-45, GPHCPB-45 (high calcium genotype)

* Corresponding author.
E-mail address: ak_gupta2k@rediffmail.com (A. Kumar).

https://doi.org/10.1016/j.gene.2018.01.071
Received 1 July 2017; Received in revised form 11 December 2017; Accepted 22 January 2018
Available online 06 February 2018

Oxalate is a major component of plants. It has been reported that oxalate biosynthesis pathway may vary from one plant species to another and involved in hydrolysis of oxaloacetate, glyoxylate oxidation, cleavage of isocitrate and oxidative cleavage of L-ascorbic acid (Hodgkinson, 1977). Also, C2/C3 cleavage of ascorbic acid results in the formation of oxalate (Libert and Franceschi, 1987). Tartaric and oxalic acids are produced by the biosynthesis and metabolism of ascorbic acid as a precursor (Loewus, 1999) and their formation differs among species. In Vitaceae family, tartarate is formed from the ascorbate by cleavage at C4/C5 carbon skeletons whereas in other species by C2/C3 cleavage, oxalate and L-threonate is formed. The L-threonate can either be converted to L-tartrate or decarboxylate to L-glycerate (Ahmad et al., 2014).

Genes involved in ascorbic acid metabolism, including Mono Dehydro Ascorbate Reductase (MHAR), functioning in both cytosol and cell wall, have been suggested to play an important role in the production of oxalic acid as well as cell expansion and tightening (Smirnoff, 1996). A major precursor of oxalate formation in rice is glyoxalate rather than ascorbate (Yu et al., 2010). These researches indicate that glyoxylate is involved in oxalate biosynthesis. The activity and distribution of glyoxylate oxidase (GLO) enzyme in oxlalate accumulating plants have not been determined yet and it is difficult to assess the contribution of this pathway to total oxalate in plants. Calcium, being important macronutrient in finger millet also possesses antinutrients such as polyphenols, phytate and oxalate (Sharma et al., 2017). Thus, it is essentially required to explore the molecular mechanism contributing to high grain calcium content and its relationship with antinutrients like oxalic acid accumulation in developing spikes of finger millet.

In the present study, attempts were made for *in silico* identification and functional characterization of oxalic acid biosynthesis pathway genes viz. (i) Serine Glyoxalate Amino-Transferase (SGAT), (ii) Glutamate Glyoxylate Amino-Transferase (GGAT), (iii) Isocitrate Lyase (ICL), (iv) Glyoxal Oxidase (GLO), (v) Mono Dehydro Ascorbate Reductase (MHAR), (vi) Ascorbate Per Oxidase (APO) and (vii) Oxalate Oxidase (OXO) using developing spikes transcriptome data of finger millet genotypes differing in grain calcium content (GP-1 and GP-45). The differential expression patterns on the basis of FPKM were validated using qPCR in finger millet genotypes. In order to establish a relationship of calcium accumulation with respect to oxalic acid and tartaric acid accumulation, contents were measured in finger millet genotypes. This study will help in elucidating the role of antinutrient factors on calcium accumulation and the molecular insights developed can be further utilized for modification of crops to raise their nutritional value and public acceptance.

2. Materials and methods

2.1. Identification and annotation of genes involved in oxalic acid biosynthesis pathway in finger millet

Developing spikes of two finger millet genotypes (GP-1 & GP-45) differing in grain calcium content were used for isolation of RNA and sequencing was done by Illumina Hi-seq (Kumar et al., 2015a). For the identification of oxalate biosynthesis pathway genes, coding sequences (CDS) of rice were retrieved. The required information of all genes with their nucleotide and protein sequences were collected from NCBI and TIGR (Rice Genome Annotation Project). Transcriptome data of two finger millet genotypes, differing in grain calcium content were used as a database and nucleotide sequences of all seven genes of rice were used as query sequences for the standalone Blastn programme. Blastn resulted in number of contigs from every particular gene with different identities and scores. Both type of contigs i.e. direct (positive) and complement (negative) were found in Blastn result. To make all the negative sequences positive, the reverse complementary tool was used for all complement genes. On the basis of lower e-value and highest score, a single contig was selected for every gene. Usually, a lower e-value indicates a better quality in the alignment, search and comparison. It is preferred over the score value because e-value is less sensitive to sequence length.

2.2. ORF prediction

Open reading frame analysis for selected contigs was done by ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) online tool. Longest frame was selected from all the given frames and their nucleotide and protein sequences were retrieved.

2.3. Phylogenetic and motif analysis of oxalate biosynthesis genes

The oxalate biosynthesis genes retrieved from finger millet transcriptome data of two genotypes were aligned with rice genes and phylogenetic tree was constructed and the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). MEGA5 standalone tool was used to construct the phylogenetic tree (Tamura et al., 2011). The percent similarity was checked through EBI online software of finger millet protein sequences along with rice (http://www.ebi.ac.uk/Tools/psa/emboss_water/protein.html). Canonical sequences conserved as block in the oxalate biosynthesis gene family were identified by using “Multiple EM for Motif Elicitation” (MEME) program (Bailey et al., 2006). Functional and structural verification of the genes was done by ScanProsite (http://prosite.expasy.org/scanprosite/) and SMART tools (http://smart.emblheidelberg.de/). The default parameters were used (with minimum width 6 and maximum width 50 amino acid).

2.4. Physicochemical properties of genes involved in oxalate biosynthesis

Physicochemical properties of protein sequences of oxalic acid biosynthesis pathway genes were predicted through Protein Identification and Analysis Tool (Protparam) on the ExPASy Server (http://web.expasy.org/protparam/). Sub-cellular localization was done by TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/). Domain analysis was done by Batch CD Search in NCBI for searching the Conserved Domain Database with protein or nucleotide query sequences (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and ScanProsite which detects PROSITE signature matches and ProRule associated structural and functional residues in proteins (http://prosite.expasy.org/). Most of the sequences are contained in the PROSITE database, so a simple PROSITE scan can give us quite revealing results.

2.5. Expression analysis of differentially expressed oxalate biosynthesis pathway genes

In the present study, sequencing reads were mapped to each gene and presented in FPKM (Fragments Per Kilobase of exon per Million fragments mapped) by using Bed Tool (Trapnell et al., 2010) for transcriptome based expression analysis. In both the GP-1 and GP-45 transcriptome, FPKM values of each contig were calculated and compared. Further R package was used to create the heat map (RDevelopment CORE, 2012).

2.6. Validation of gene expression through quantitative PCR

For further analysis using RT-PCR, plants were grown in polyhouse under controlled condition and total RNA was isolated from four stages of developing spikes (S1 – spike emergence; S2 – pollination stage; S3 – dough stage and S4 – maturation stage) using RNA isolation iRIS system from IHBT Palampur. To remove residual DNA contamination, RNA was treated with RNase-free DNase I for 30 min at 37 °C. Total 2μg of RNA was used for the first-strand cDNA synthesis using Revert Aid H-minus reverse transcriptase cDNA synthesis kit (Fermentas, Germany).