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Original article

Cloning, expression and molecular modeling of the anthocyanidin reductase (*FaANR*) gene during strawberry fruit development

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Summary

Introduction – **Proanthocyanidins (PAs) are a group of polyphenolic secondary metabolites synthesized in plants via flavonoid pathway. Strawberry (*Fragaria* × *ananassa* Duch.) is a rich source of flavonoids and proanthocyanins, which are known to have multiple health benefits. The anthocyanidin reductase (*ANR*) is an interesting gene to study within the flavonoid biosynthesis pathway, since it diverts the anthocyanin pathway to flavonol synthesis.** *Materials and methods* – **The present study describes cloning, semi-quantitative expression analysis and molecular modeling of the strawberry (cv. Sweet Charlie) anthocyanidin reductase (*FaANR*) gene during the progressive stages of fruit development.** *Results and discussion* – **The *FaANR* gene was 1,020 bp long with an open reading frame encoding a protein of 308 amino acid residues. The estimated molecular mass and isoelectric point of the protein were 32.89 kD and 5.54, respectively. The expression of *FaANR* was only seen during early stages of fruit development indicating its early involvement in PA accumulation, well before ripening onset. Analysis of the *FaANR* sequence showed 98% similarity to *ANR* from diploid strawberry, *Fragaria vesca*. The cladistic analysis indicated that the *FaANR* was phylogenetically similar to *Pyrus* and *Prunus ANR* genes. Protein modeling suggested protein-ligand interactions at active site with NADP binding as the plausible mechanism of action.** *Conclusion* – **This is the first report on cloning, expression study and *in silico* modeling of an anthocyanidin reductase of strawberry. The conditions of *in vivo* modulation of *ANR* expression open applied perspectives for commercial production of PAs in strawberry and other berries.**

Keywords

strawberry, *Fragaria* spp., phenolics, flavonoid biosynthesis pathway, homology modeling, molecular docking

Abbreviations

ANR: Anthocyanidin reductase; CTs: Condensed tannins; DAF: Days after flowering; MSL: Mean sea level;

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Significance of this study

What is already known on this subject?

- Strawberry fruits are rich in anthocyanin, proanthocyanidin and condensed tannins.

What are the new findings?

- This is the first report on cloning, expression analysis and *in silico* protein modeling of the *FaANR* gene.

What is the expected impact on horticulture?

- The study has identified a precise stage for *in vivo* modulation of *ANR* expression to improve the strawberry proanthocyanidin status.

PAs: Proanthocyanidins; SOC: Super Optimal broth with Catabolite repression; UFGT: UDP-glucose:flavonoid 3-O-glucosyltransferase

Résumé

Clonage, expression and modélisation moléculaire du gène codant pour l'anthocyanidine réductase (*FaANR*) au cours du développement de la fraise.

Introduction – **Les proanthocyanidines (PA) sont un groupe de métabolites secondaires polyphénoliques synthétisés dans les plantes via la voie des flavonoïdes. La fraise (*Fragaria* × *ananassa* Duch.) est une riche source de flavonoïdes et de pro-anthocyanes, qui sont connus pour avoir de multiples bénéfices pour la santé. L'anthocyanidine réductase (ANR) est un gène intéressant à étudier dans la voie de la biosynthèse des flavonoïdes, puisqu'elle détourne la voie anthocyanique vers la synthèse de flavonol.** *Matériel et méthodes* – **La présente étude décrit le clonage, l'analyse d'expression semi-quantitative et la modélisation moléculaire du gène de l'anthocyanidine réductase fraise (cv. Sweet Charlie) pendant les étapes progressives du développement des fruits.** *Résultats et discussion* – **Le gène *FaANR* mesure 1.020 pb de long avec un cadre de lecture ouvert codant pour une protéine de 308 résidus d'acides aminés. La masse moléculaire estimée et le point isoélectrique de la**

protéine sont de 32,89 kD et 5,54, respectivement. L'expression de *FaANR* n'a été observée que durant les premiers stades du développement des fruits, ce qui indique son implication précoce dans l'accumulation de PA, bien avant le début de la maturation. L'analyse de la séquence *FaANR* a montré une similitude de 98% avec l'*ANR* de la fraise diploïde *Fragaria vesca*. L'analyse cladistique a indiqué que le *FaANR* était phylogénétiquement similaire aux gènes *ANR* de *Pyrus* et de *Prunus*. La modélisation des protéines suggère des interactions protéine-ligand au site actif avec la liaison au NADP comme mécanisme d'action plausible. *Conclusion* **– Il s'agit du premier rapport de clonage, d'étude d'expression et de modélisation *in silico* de l'anthocyanidine réductase de fraise diploïde. Les conditions de modulation *in vivo* de l'expression de l'*ANR* ouvrent des perspectives appliquées pour la production commerciale d'AP dans la fraise et d'autres baies.**

Mots-clés
fraise, *Fragaria* spp., composés phénoliques, voies de biosynthèse des flavonoïdes, modélisation par homologie, couplage moléculaire

Introduction

Flavonoids are a large family of secondary metabolites widely produced by plants. They have been associated with a range of functions from protection against herbivory and pathogen attack to a response towards environmental stresses like ultraviolet (UV) radiation and reactive oxygen species (Gould and Lister, 2006). Further, their involvement in plant reproduction (Mo *et al*., 1992), symbiosis (Wasson *et al*., 2006) and signaling (Buer *et al*., 2006) has also been demonstrated. Flavonoids influence anthocyanin color via co-pigmentation in the vacuole (Fossen *et al*., 2000; Lancaster, 1992). The anthocyanidin reductase (*ANR,* E.C. 1.3.1.77) converts cyanidin to epicatechin (2,3-*cis*-flavanols) via a NAPDH-mediated reduction. Epicatechin is reported to have insulin mimetic actions with protective effects on erythrocytes in a manner similar to insulin (Rizvi and Zaid, 2001). These compounds also offer antioxidant protection against lipid peroxidation and inhibit platelet aggregation (Mandave *et al*., 2014).

In recent years, PAs and their monomers have received considerable attention because of their potential health benefits such as immunomodulatory and anticancer activities (Zhao *et al*., 2009), antioxidant and free radical scavenging functions (Mandave *et al*., 2013, 2014; Prior and Gu, 2005; Rani *et al*., 2014), anti-inflammatory activities (Subarnas and Wagner, 2000), cardio-protective properties (Sato *et al*., 1999), vasodilating and antithrombotic effects (Sano *et al*., 2005) and UV-protective functions (Sharma *et al*., 2007). Their inclusion in human diet is therefore significantly important as nutraceutic agents (Lo Piero *et al*., 2005).

Strawberry (*Fragaria* × *ananassa* Duch.) is a commercially important soft fruit with flavor as a vital quality factor. The fruits are popular due to soft-texture, attractive aroma, pleasant flavor and nutrient content (Hancock, 1999). Metabolic changes that occur during fruit maturation have direct impact on the formation of compounds associated with these traits. Fruit growth and ripening are physiologically extremely dynamic processes including degradation of chlorophyll, accumulation of anthocyanins, softening of fruits, metabolism of organic acids and sugars, and production of flavor compounds. Although the pigments from strawberry fruits have been partly characterized (Lopes-da-Silva *et al*., 2000, 2007), the anthocyanin-synthesis pathway in strawberry still remains poorly understood.

Xie *et al*. (2003), reported the activity of anthocyanin reductase (*ANR*), *i.e*., the *BANYULS* (*BAN*) genes from *Arabidopsis thaliana* and *Medicago truncatula*, which convert anthocyanidins into their corresponding 2,3-*cis*-flavan-3-ols. Ectopic expression of BAN in tobacco flower petals and *Arabidopsis* leaves results in loss of anthocyanins and accumulation of condensed tannins (CTs). The expression and cloning of *ANR* has been reported from grapes (Bogs *et al*., 2005; Fujita *et al*., 2005; Gargouri *et al*., 2010) and green tea (Zhang *et al*., 2012) indicating early expression of the gene during fruit development.

Though strawberry fruits are well-known for higher level of anthocyanin, PAs and CT, there is no report on *ANR* gene from cultivated octaploid strawberry. Further, the expression pattern of strawberry *ANR* during fruit development and its protein structure is still unknown. The functionally critical location of *ANR* gene in the flavonoid biosynthesis pathway leading to diversion towards flavonol synthesis makes it an attractive candidate for structural and functional characterization. Knowledge of *ANR* gene expression with respect to fruit development is critical for improving strawberry fruit quality and nutrition. In this study, we report cloning, *in silico* characterization and homology modeling of *Fragaria* × *ananassa Anthocyanidin reductase (FaANR)* and its expression pattern at progressive stages of strawberry (*Fragaria* × *ananassa* Duch. cv. Sweet Charlie) fruit development. To the best of our knowledge, this is the first report of cloning, expression studies and homology modeling of *ANR* from strawberry.

Materials and methods

Plant material collection

Strawberry (*Fragaria* × *ananassa* Duch.) cv. Sweet Charlie fruit was collected from Mahabaleshwar (17.98°N, 73.67°E), Maharashtra, India during the 2011–12 season. Mahabaleshwar has an average elevation of 1,384 m above the mean sea level. The fruits were harvested in the morning, collected from the field after flowering and thereafter at every 4 days (Figure 1) throughout the berry development stages until maturity (DAF 0, 4, 8, 12 and 16). The fruits were randomly collected (flowers tagged and fruits were collected from selected plant randomly), frozen in liquid nitrogen and stored at -80 °C until further analysis.

RNA isolation and synthesis of cDNA

Total RNA was isolated from developing fruit at different growth stages using Total Plant RNA extraction kit (Sigma) according to the manufacturer protocol. Approximately, 100 mg tissue was used for RNA isolation. After DNase treatment, RNA was quantified using ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). For cDNA synthesis, RNA was reverse transcribed using oligo (dT) and Super-Script III Reverse Transcriptase (Invitrogen) as per the supplier's protocol. The cDNA was normalized using 18S housekeeping primers (Gigliotti *et al*., 2004; Maroufi *et al*., 2010). The sequence of the primers are F- 5'CCGGTCCGCCTATGGTGTGCACCGG-3' and R-5'CCTCTGACTATGAAATACGAATGCCCC-3'.

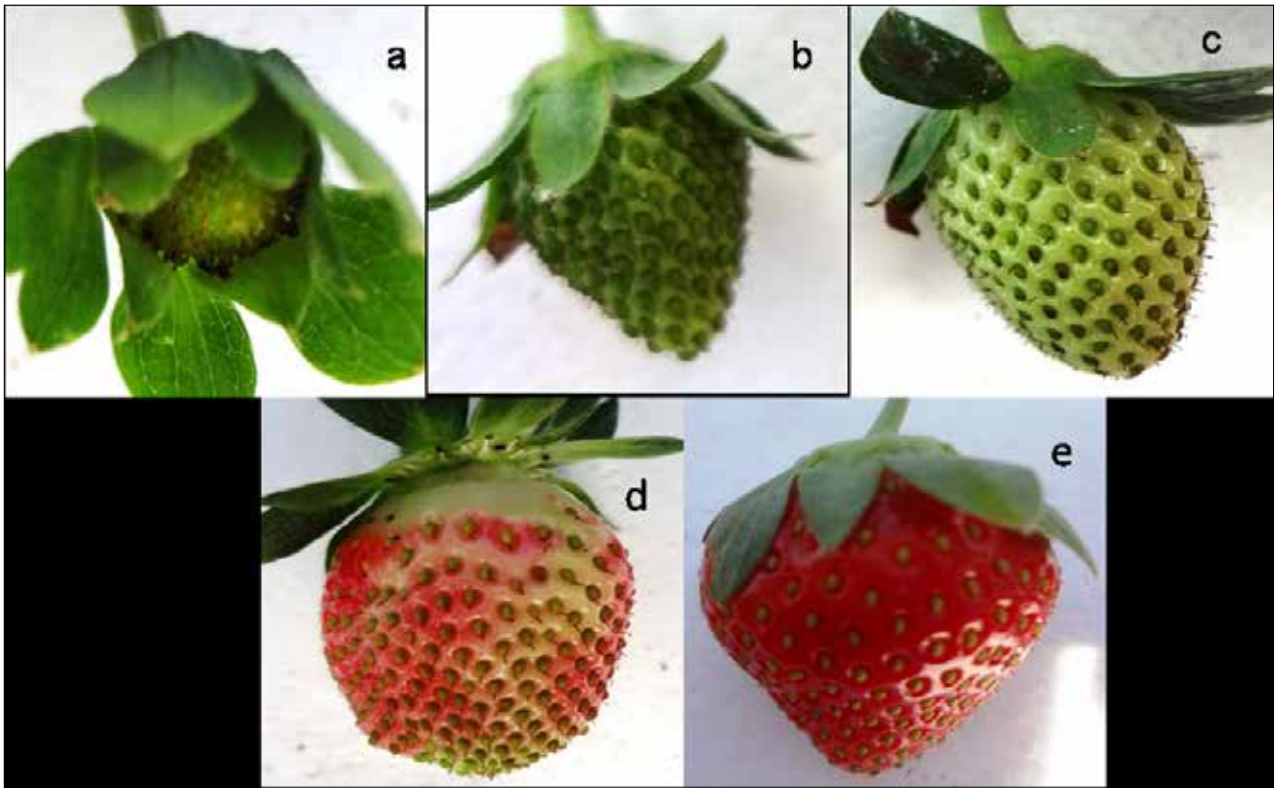


Figure 1. Cultivar Sweet Charlie strawberry fruit at different developmental stages (a, b, c, d and e at 0, 4, 8, 12, 16 days after flowering (DAF), respectively.

Primer designing

Strawberry Anthocyanin reductase (*ANR*) sequences from NCBI (NCBI Accession numbers DQ664192.1, DQ438979.1, HM026188.1 and DQ664193.1) were aligned in MEGA4 (version 4.0.2) and conserved regions were detected. The conserved region was used to design primers for semi-quantitative PCR, using PrimerPremiere (version 3.0) with default settings. The best possible primers were selected on the basis of GC content, Tm, secondary structures and self/false priming. The sequence of the primers are F-5'ATAAGGTACCGCGATGTCCACCACC-3' and R-5'CGTTCCCGGGTTTCTAGTTCTGCAG-3'.

PCR amplification of *FaANR*

The generated cDNA (1:40 diluted; 75 pg) was used as template for PCR amplification (Gene Amp®PCR system 9700, Applied Biosystems, US). The PCR conditions were set for initial denaturation at 94 °C for 10 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 30 sec and extension at 72 °C for 1 min; followed by final extension at 72 °C for 10 min. PCR products were resolved on 0.8% agarose gel, stained with ethidium bromide and visualized under UV transilluminator (Alphaimager EC). The PCR product was purified from the gel using Quick gel extraction kit (Invitrogen) and eluted in 30 µl elution buffer according to manufacturer guidelines.

Cloning of *FaANR*

The purified PCR products were used for cloning. The putative *FaANR* was ligated in pGEM®-T Easy Vector (Promega) as per manufacturer's protocol. The ligated products were used for transformation of ultra-competent *E. coli* (Top 10) using heat shock method (Froger and Hall, 2007). Transformants were selected on agar LB media supplemented with Ampicillin, IPTG and X-Gal for blue/white screening. The white colonies were selected and cultured in LB medium supplemented with ampicillin at 37 °C overnight.

Plasmid isolation and restriction digestion

Plasmids were isolated from overnight grown cultures of transformed cells using Plasmid Isolation Miniprep Kit (Axygen) as per the manufacturer's instructions. The plasmid DNA was eluted in 60 µL elution buffer, quantified and was subjected to restriction digestion using *Eco*RI. The uncut and restriction digested plasmid DNA were visualized using 0.8% agarose gels. Plasmid DNA from transformed colonies was sequenced by Sanger sequencing.

Sequence analysis

The putative *FaANR* sequence was screened using VecScreen tool (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) to eliminate any vector sequences. The edited sequences were characterized using 'blastn' tool from NCBIBLAST search (http://blast.ncbi.nlm.nih.gov/). The similar sequences were downloaded and subjected to phylogenetic analysis. Further, the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to detect ORFs within the putative *FaANR* sequences and the ORFs were analyzed to detect conserved protein sequence domains. ClustalW (2.0.11) was used for multiple sequence alignments. The phylogenetic reconstruction was performed by neighbor-joining method (Saitou and Nei, 1987) with 1,000 bootstraps using Phylogeny Inference Package (PHYLIP) in MEGA 4 version 4.0.2 (Tamura *et al.*, 2007).

Gene expression analysis during fruit development

Temporal gene expression patterns were analyzed by semi-quantitative PCR. The PCR conditions were the same as mentioned above. *FaANR* specific primers and cDNAs from various fruit development stages (DAF 0, 4, 8, 12 and 16) were used for the study.

Modeling of *FaANR*

The *in silico* translated amino acid sequence of *FaANR* was used to generate 3D structure of the protein. The amino acid sequence was analyzed using ProCheck and the proteins with maximum identity (*viz*. Anthocyanidin reductase from *Vitis vinifera*, PDB ID: 2RH8, Putative Acetoacetyl-CoA reductase from *Burkholderia cenocepacia*, PDB ID: 4K6F, and putative Mevalonate kinase from *Leishmania major*, PDB ID: 2HFS) were selected as template molecules for alignment with query sequence. The structures of the template molecules were obtained from Protein Data Bank (PDB). The Modeller 9.11 (Sali and Blundell, 1993), I –TASSER (Roy *et al*., 2010), Phyre2 Web Server (Kelley and Sternberg, 2009) and SWISS MODEL (Schwede *et al*., 2003) were used to generate 3D structure of *FaANR*. A validated and most stable structure was obtained from Modeller9.11 (Olsson *et al*., 2011) and was used for molecular dynamics and molecular docking analysis.

Refinement of homology model

Molecular dynamics simulations were performed to optimize the obtained model. The protonation states of the amino acid residues were determined at pH 7.0 by PROPKA (Schwinn *et al*., 2006) web server. Prior to molecular dynamics simulation, the peptide had its geometry energy minimized using a steepest descent algorithm, followed by conjugate gradient algorithm. Low temperature replicas (decoys) generated during the simulation were clustered by SPICKER and top five cluster centroids were selected for generating full atomic models.

Molecular docking

Molecular docking was performed to validate the protein model in which ligand interacts with the protein target. Based on the available literature for *ANR*, NADP Nicotinamide-Adenine-Dinucleotide Phosphate (PDB ID: 4K6F) was selected ligand for optimized *ANR* protein receptor. The "Ligand-Fit of receptor-ligand interactions protocol" was selected for docking in Discovery Studio 1.7, all duplicate structures were removed and ionization change, tautomer generation, isomer generation, Lipinski filter, and 3D generator were set. For receptor preparation, hydrogen molecules were added to the protein molecules (PDB ID: 4K6F) and associated water molecules were removed.

The pH of the protein was set in the range of 6.5–8.5 and the active site was selected based on the ligand binding domain. LigandFit was chosen for receptor-ligand interaction and PLP1 was selected as the energy grid. The conformational search of ligand poses was performed by Monte Carlo method with the torsional step size for polar hydrogen set to ten. The docking was performed with consideration of electrostatic energy with maximum internal energy of 10,000 Cal. However, no attempt was made to minimize the ligand-receptor complex (rigid docking). After completion of docking, the docked enzyme (protein–ligand complex) was analyzed to investigate the type of interactions. Docking conformers of each compound were ranked according to their dock score function. The original bound ligand was extracted and docked to the generated 3D structure of *ANR* and binding interaction with the ligand was compared.

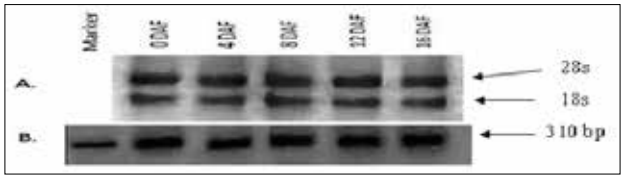
Results and discussion

The biosynthetic pathways of condensed tannins and flavonoids are the most characterized secondary metabolic routes (Schwinn *et al*., 2006). The biosynthesis starts from phenylalanine to produce phenylpropanoids that are subsequently channeled into the flavonoid pathway by various stage specific enzymes leading to the synthesis of anthocyanidin and proanthocyanidin (PA) pigments. Flavonol synthase (FLS), leucoanthocyanidins reductase (LAR) and Anthocyanidin reductase (*ANR*) play important roles in diverting the biosynthetic pathway from anthocyanins to flavonols. *ANR* is not only important for PA synthesis, but it also influences the synthesis of anthocyanidin by competing with UFGT (UDP-glucose:flavonoid 3-O-glucosyltransferase) through which it converts anthocyanidin to anthocyanin (Bogs *et al*., 2005).

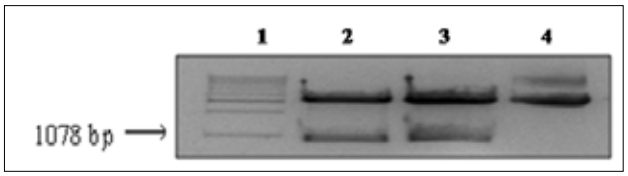
The present study describes isolation of the full length *FaANR* gene from strawberry cv. Sweet Charlie and its expression pattern during fruit developmental stages. We are also reporting the homology modeling and docking of the same molecules.

Characterization of *FaANR* cDNA and its temporal expression

The total RNA from strawberry fruits at five different development stages and cDNA normalized using the 18S primers is shown in Supplementary Figure 1. The restriction digestion analysis of the cloned *FaANR* plasmid by *Eco* R1 produced a DNA band of expected size, 1,020 bp (Supplementary Figure 2).



Supplementary Figure 1. The total RNA from strawberry fruits at five different development stages and cDNA normalized using the 18S primers.



Supplementary Figure 2. The restriction digestion analysis of the cloned *FaANR* plasmid by *Eco*R1.

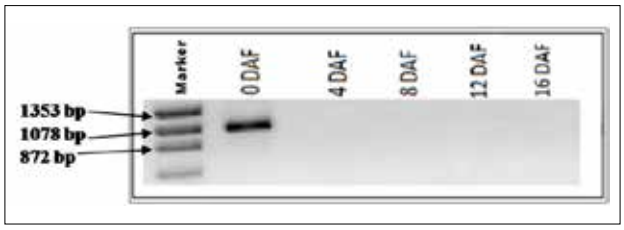


Figure 2. Analysis of temporal expression pattern of anthocyanidin reductase gene during progressive fruit developmental. Marker: phi X 174/Hind III digest Molecular Marker.

The *FaANR* cDNA was found to be 1,020 bp long with an open reading frame encoding a protein of 308 amino acid residues. The sequence is available from NCBI with the accession number JX271492. The *in silico* analysis of *FaANR* using BLASTN revealed 98% sequence identity at the nucleotide level with *ANR* gene from other strawberry varieties, *i.e.*, 'Queen Elisa' (DQ664192.1) and 'Korona' (DQ438979.1) and, to a lesser extent (83%), to the *ANR* from *Pyrus communis* (DQ251189.1). The BLASTP analysis also showed 98% identity at the amino acid level to *ANR* from 'Queen Elisa' (ABG76842.1 and ABG76843.1) and 'Korona' (ABD95362.1).

After characterization of *FaANR* cDNA, we analyzed its expression pattern during five fruit development stages. Figure 2 shows temporal expression (semi-quantitative expression levels) of *FaANR* during progressive fruit developmental stages (DAF 0, 4, 8, 12 and 16). Interestingly, the expression of *FaANR* was observed only at 0 DAF stage which is a quite early stage of fruit development. The amount and composition of PAs varies in different parts of the fruit and at different developmental stages of the berry (Downey *et al*., 2003; Kennedy *et al*., 2000). Proanthocyanidins have been reported to be synthesized during the first phase of grape berry development, and there is a decline in extractable PAs after the onset of ripening and throughout ripening, which is thought to be the result of complexation of the PA polymers with other cellular components (Kennedy *et al*., 2000, 2001; Xie *et al*., 2006). The initial higher concentration of PAs (Davood and Osamu, 2006; Whale and Singh, 2007) may thus be explained on the basis of expression of *FaANR* limited to earlier stages of fruit development in apple. Our data is in accordance with the previous reports in other fruits such as apple (Davood and Osamu, 2006; Whale and Singh, 2007) what indicates the expression of *ANR* at early developmental stages of the fruit. Condensed tannins and proanthocyanidins are the main compounds involved in plant defense (Feeny, 1970; Scalbert, 1991; Bais *et al*., 2003; Koes *et al*., 2005) and synthesized by the *ANR* gene (He *et al*., 2008). Higher expression of *ANR* at early fruit development stage could be responsible for this activity.

The estimated molecular mass and isoelectric point of the protein was 32.89 kD and 5.54, respectively. Total number of negatively charged and positively charged residues were 34 and 27, respectively. Under *in silico* mimicked physiological conditions, the protein was found to be stable and had an instability index of 28.40.

Phylogenetic analysis of *FaANR*

Phylogenetic reconstruction of ANR sequence similarity to *FaANR* was attempted to detect its evolutionary placement and phylogenetic similarity with other similar genes. In the nucleotide BLAST, the isolated strawberry *FaANR* gene showed maximum (≥ 75%) similarity with *Pyrus communis* (83%), *Prunus avium* (84%), *Vitis bellula* (79%), *Populus trichocarpa* (77%) and *Ricinus communis* (76%). So, these five plants nucleotide sequences were used for the phylogenetic analysis and amino acid alignment. The phylogenetic tree (Figure 3) based on sequence similarities can be divided into two major clades. These two clades represent three orders of subclass Rosidae *viz*. Malpighiales, Rhamnales, Rosales. Clade I bifurcates into two groups. Plants under order Rosales, family Rosaceae (*Pyrus communis, Prunus avium* and *Fragaria* × *ananassa*) form one group whilst as *Vitis bellula* (family Vitaceae) forms a separate unit. Plants in the second clade belong to order Malpighiales (*Populus trichocarpa* and *Ricinus communis*). Plants belonging to clade I, are known to accumulate flavonoids like anthocyanidins and proanthocyanidins. Among them, genera *Prunus* and *Fragaria* × *ananassa* show maximum accumulation of these compounds (Treutter, 1988; Mouradov and Spangenberg, 2014). Till now the *FaANR* from strawberry has not been phylogenetically analyzed. Many reports are available from other plants species. Phylogenetic analysis of ANR from grape and *Arabidopsis* has been extensively reported (Bogs *et al*., 2005). Grape ANR is ortholog with the *Arabidopsis*, *Gossypium arboretum* and *Medicaga truncatula*.

Protein (amino acid) alignments of *FaANR*

The conserved domain and protein similarity between the *ANR* from strawberry and other plant species were analysed. The ORF corresponding to *FaANR* coded for protein with 308 amino acids. *In silico* amino acid sequence were generated from *Pyrus communis*, *Prunus avium*, *Vitis bellula, Populus trichocarpa* and *Ricinus communis*. These five plants showed maximum (≥ 75) BLAST similarity with isolated FaANR. The putative *FaANR* sequence was compared with protein from phylogenetically similar plants (Figure 4). The figure depicts the conserved domain of ANR Rossmann fold, NADPH – binding regions and a structurally diverse C-terminal region.

Anthocyanidin reductase belongs to oxidoreductase enzyme family and extended SDR (eSDR) subgroup. Such flavo-

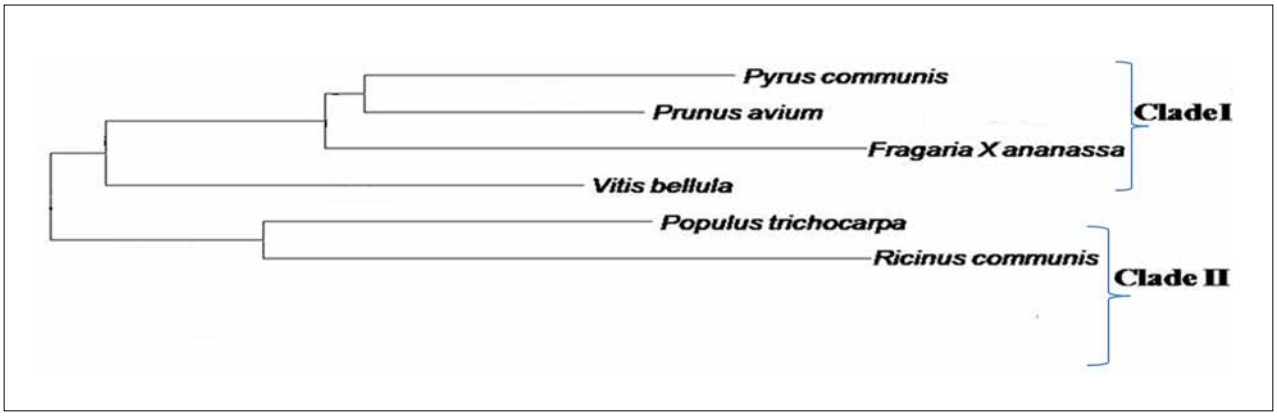


Figure 3. Phylogenetic analysis of the *FaANR* gene with other genes. Phylogenetic tree deduced from the multiple sequence alignment of nucleotides homologous to *FaANR* from different plant species. Gene bank accession numbers are as follows: *Pyrus communis*: DQ251189.1, *Prunus avium*: GU938688.1, *Fragaria* × *ananassa*: JQ271492.1, *Vitis bellula*: JQ308620.1, *Populus trichocarpa*: XM_002317234.1 and *Ricinus communis*: XM_002518532.1. Numbers indicate bootstrap values.

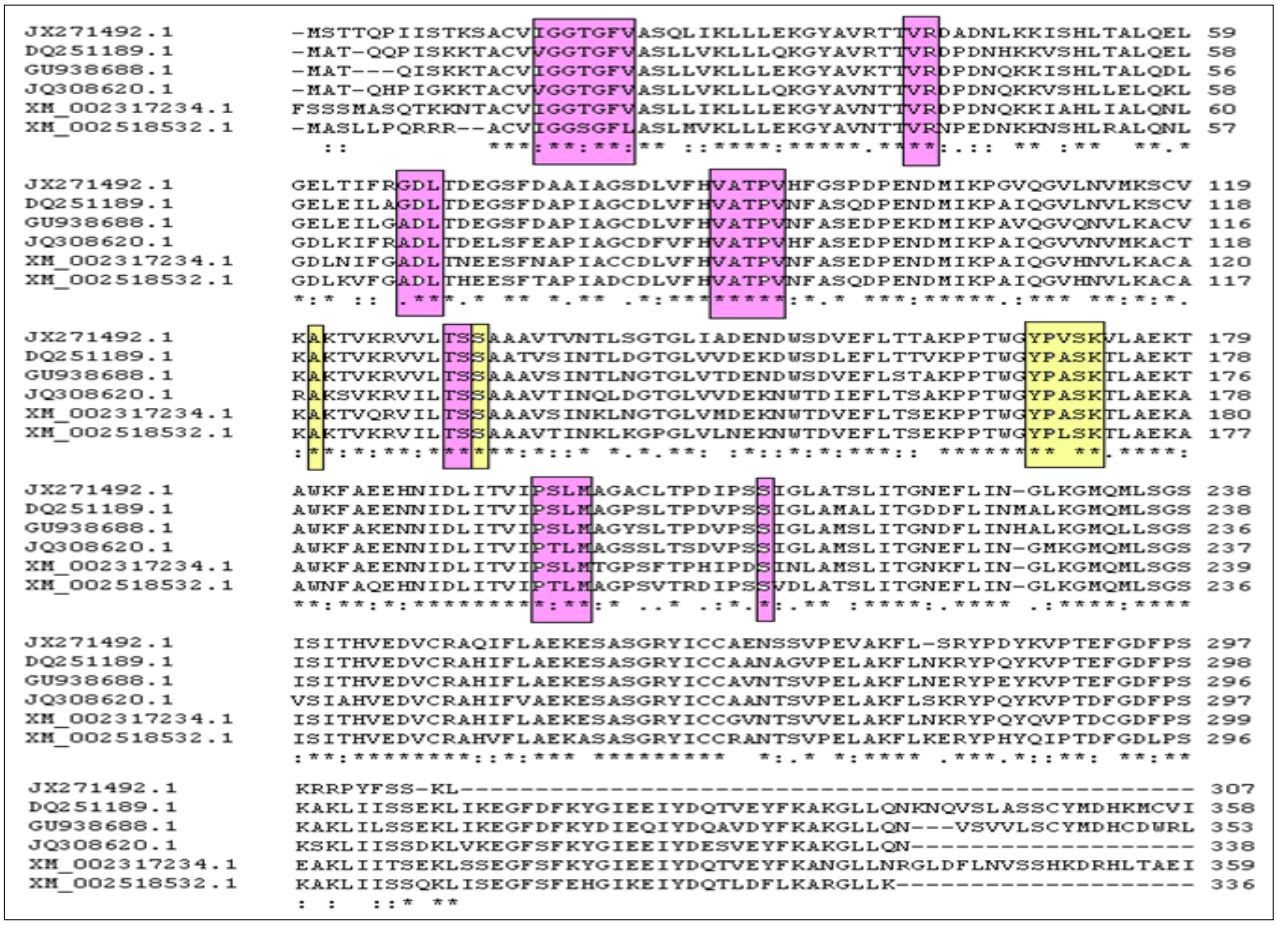


Figure 4. Amino acid alignment of the *FaANR* with five other ANR proteins related plants. Comparisons of the deduced amino acid sequence of the putative *FaANR* gene with five related plant ANR proteins. Alignment was carried out using ClustalW. The related protein sequence aligned are as follows (GeneBank accession number): *Pyrus communis* (DQ251189.1), *Prunus avium* (GU938688.1), *Fragaria* × *ananassa* (JQ271492.1), *Vitis bellula* (JQ308620.1), *Populus trichocarpa* (XM_002317234.1), *Ricinus communis* (XM_002518532.1). Yellow shaded part is associated with the active sites of the enzyme and pink shaded part codes for the NADP binding sites.

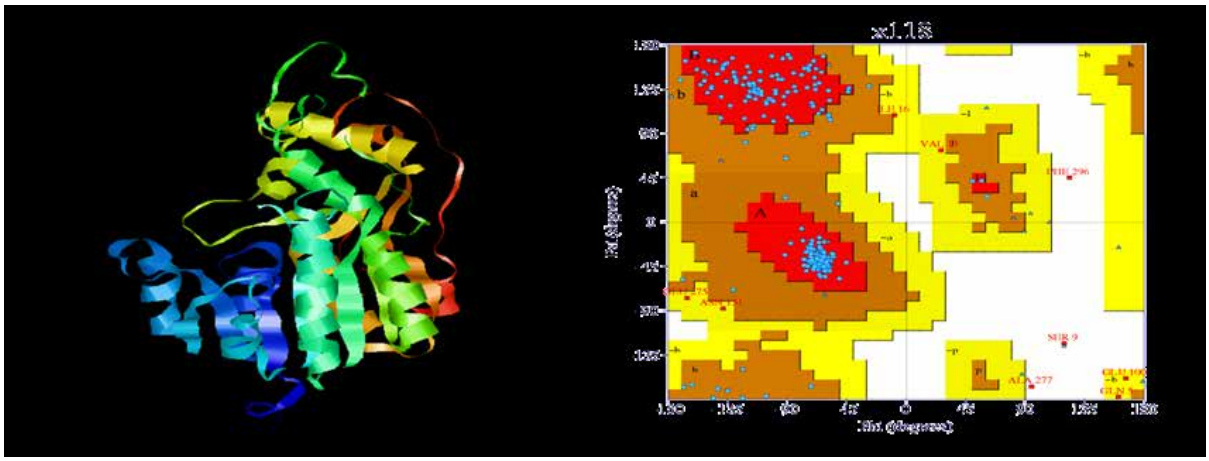


Figure 5. Optimized model and Ramachandran plot of the *FaANR*. The optimized low energy model was developed using Modeller 9.11. The Ramachandran plot was determined by PDBsum tool (PROCHECK).

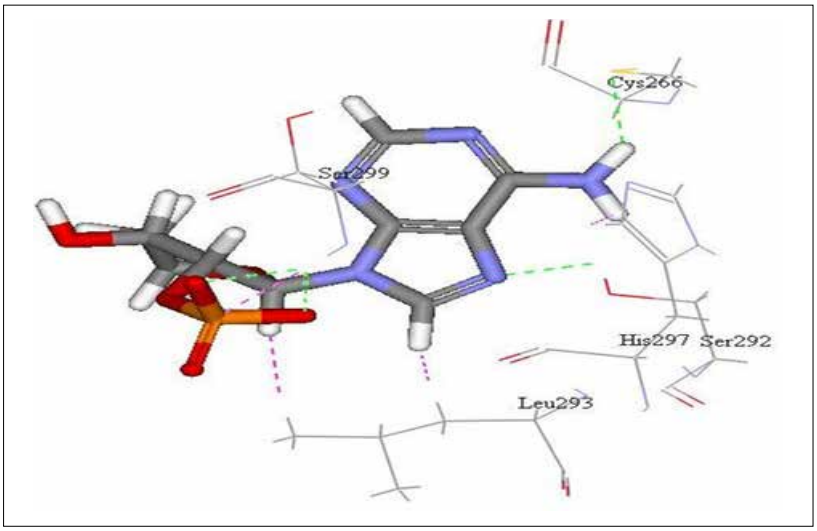


Figure 6. Protein-ligand docking showing molecular interaction with each other.

nol reductases act through NADP-dependent reduction of flavonoids and have characteristic active site triad of SDRs and a NADP-binding motif. SDRs are a functionally diverse family of oxidoreductases that have a single domain with a structurally conserved Rossmann fold, an NAD(P)(H)-binding region, and a structurally diverse C-terminal region (pink shaded square). Classical or extended SDRs have YXXXK active site motif (yellow shaded square), with the tyrosine residue of the active site motif serving as a critical catalytic residue. In addition to tyrosine and lysine, there is often an upstream serine and/or an asparagine, contributing to the active site; while substrate binding is in the C-terminal region determining the specificity.

Homology modeling and molecular docking

After protein alignment to identify conserved domains, we performed homology modeling. For this, the protein structure was deduced and the activity assessed by successful docking with the NADP binding site. Figure 5 depicts the low energy molecule of *FaANR* along with Ramachandran plot. Ramachandran plot revealed that 91.5% (280/306) of all residues were in favored region, 5.22% (16/306) residues were in allowed regions, and remaining 2.77% (10) residues were outliers (phi, psi).The protonation state of the protein was determined using PROPKA tool. The pH with optimum stability was 3.7 for which the free energy was found to be 36.8 kcal mol^{-1} at 298 °K. This model was validated using the Swiss-model online tool. The dihedrals, covalent and overall G-factors were -0.11, -0.86 and -0.39, respectively.

Molecular docking study was performed on 3D structure of *ANR* obtained from homology modeling technique to check binding interaction of template molecule (PDB ID: 4K6F) with bound ligand (NADP). Ligand-protein interactions of template molecule indicated that polar amino acid residues *viz*. Ser299, Cys265, His297, Ser292 and non-polar Leu293 formed active binding interactions with ligand forming catalytic residues. The docked pose of most favorable conformer is shown in Figure 6. The best docked pose revealed the same amino acid residues at the active site as the template which are crucial for binding interactions. The NADP molecule interacts in same fashion as in case of template molecule.

A model for applied perspectives

FaANR plays a key role in the production of PAs, a group of secondary metabolites with great commercial interest as nutraceuticals. Their complex structure limits the commercial chemical synthesis and we are therefore highly reliant on plants, especially berries, as primary source of PAs. Proanthocyanidins have been reported to possess multiple health promoting and disease-preventing properties (Nair *et al*., 2004). Therefore, they are generally added into foods (functional foods), prescribed as supplements, or both (Lanchance, 2004). It has been reported that anthocyanins are undetectable in green and white fruits and that their levels increase rapidly during berry maturation (Cheng and Breen, 1991; Given *et al*., 1988). In contrast, the concentrations of polyphenols and non-tannin flavonoids reach their maximum in green fruits and decrease thereafter (Cheng and Breen, 1991; Wang and Lin, 2000). Phenylalanine ammonia-lyase (PAL) is the first enzyme involved the anthocyanidins and proanthocyanidins biosynthesis (Cheng and Breen, 1991; Given *et al*., 1988). The *PAL* gene shows two expression peaks during strawberry fruit development, *i.e*., at early and late fruit development stages. The first peak of expression corresponds to the accumulation of flavonoid while the second peak corresponds to anthocyanidin accumulation (Cheng and Breen, 1991). The current study was aimed at isolating and characterizing strawberry *ANR* to imagine the production of commercial quantities of PAs in other plant systems in the future. Plants such as bilberry, blackberry, blueberry, cherry, *etc*., produce high levels of cyanidins which can be diverted for the production of PAs by introduction of a suitable *ANR* gene. *In vivo* modulation of gene expression to regulate anthocyanin accumulation in different fruit crops, although challenging, is of enormous commercial value (Xie *et al*., 2011).

Conclusion

To our knowledge, this is the first report on cloning of *FaANR*, on its expression analysis under five fruit development stages and *in silico* protein modeling. Phylogenetically, this gene shows similarity (1 to 11%) with *Pyrus*, *Prunus*, *Vitis*, *Populus* and *Ricinus* species. Protein alignment with *ANR* from these plants revealed NADP binding site as conserved domain and the molecular docking supported these results. Further structural analysis with the help of X-ray crystallography or NMR spectroscopy needs to be undertaken to unravel the structural details of this protein. This study has identified a precise stage at which the attempts for *in vivo* modulation of *ANR* expression can be undertaken to improve

the PA content of strawberry and other berry fruits in which complex structures of PAs limit accessibility to their commercial production. The present work may serve as a prototype for similar studies in other berries and modulation of *ANR* genes for commercial production of PAs.

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