STUDY OF ROOT ARCHITECTURE MODIFICATION IN WHEAT: BIOINFORMATIC AND MOLECULAR APPROACHES

Ph.D. THESIS

by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in this thesis entitled **"STUDY OF ROOT ARCHITECTURE MODIFICATION IN WHEAT: BIOINFORMATIC AND MOLECULAR APPROACHES"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2011 to July, 2016 under the supervision of Dr. G.S. Randhawa, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(DEEPA DEWAN)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

(G.S. Randhawa)
Supervisor

The Ph. D. Viva-Voce Examination of **Ms. Deepa Dewan**, Research Scholar, has been held on .....

Chairman, SRC **Signature of External Examiner**

This is to certify that the student has made all the corrections in the thesis.

Signature of Supervisor **Head of the Department**
Dated: _____

Dedicated

to my Beloved Parents

ABSTRACT

Root architecture is considered as an important agronomic trait for yield development in crops. Despite the global importance of wheat, little information is available on the molecular mechanism of root architecture modification in response to nitrate stress. To understand the molecular mechanism of root architecture in response to nitrate stress, the root architecture traits of eight commercial wheat cultivars at various concentrations of nitrate were tested. Two cultivars, WH1021 and PBW343, were selected as a nitrate stress tolerant cultivar and a nitrate stress sensitive cultivar, respectively for further studies. The specific activities of two antioxidant enzymes, namely, peroxidase and superoxide dismutase, were found increased in the nitrate stress tolerant cultivar WH1021 under nitrate stress. By designing the primers from 4 root architecture related genes, namely, *TaEXP*, *TaGLU3*, *TaNRT2* and *TaMnSOD*, root architecture related genes in wheat were amplified by polymerase chain reaction (PCR) technique. The reverse transcriptase (RT) PCR results revealed that except *TaMnSOD*, the expression of all the other gene was down-regulated in the roots of nitrate tolerant cultivar WH1021 seedlings under nitrate stress. Identification and functional characterization of rootless concerning crown and seminal roots (*RTCS*) gene in wheat was also done. The amino acid conservation of RTCS protein was studied in six monocot (*Zea mays, Sorghum bicolour*, *Setaria italica*, *Oryza sativa*, *Panicum virgatum* and *Triticum aestivum*,) and six dicot (*Arabidopsis thaliana*, *Solanum tuberosum*, *Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) species of plants. The results revealed that RTCS protein consists of conserved LOB domain in all the 12 plant species. It was also found that all monocot gene sequences of *RTCS* carried CpG islands at both 5' and 3' ends. The *in-silico* physical mapping results indicated the presence of *TaRTCS* gene on chromosomes 4A, 4B and 4D of wheat. The absence of PCR amplification in the deletions lines of chromosome 4 of wheat confirmed the results obtained by the *in-silico* physical mapping. Semi-quantitative PCR analysis showed the expression of Ta*RTCS* gene to be highest in seedling roots (6.5 folds) followed by germinating roots (5 folds) and crown (3.8 folds) normalized to actin. Virus induced gene silencing (VIGS) of *TaRTCS* gene using barley stripe mosaic virus (BSMV) was done to functionally characterize this gene in Chinese spring cultivar of wheat. The phenotype obtained after the inoculation with infectious virus containing γ.Ta*RTCS* construct showed 30% decrease in the root length as compared to the control plants. The real time PCR results showed 66% decrease in RTCS transcripts level in the seedling roots inoculated with infectious virus

containing γ.Ta*RTCS* construct as compared to the control plants. The expression of *TaRTCS* gene was found to be maximum (6.2 folds) after the 6 h exposure with indole 3-acetic acid. These findings are expected to be helpful in understanding the molecular mechanism of root architecture modification in wheat.

Keywords: Wheat, Root architecture, Rootless concerning crown and seminal roots, Virus induced gene silencing

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(Deepa Dewan)

ABBREVIATIONS

%	:	Percent sign
˚C	:	Degree centigrade
€	:	Molar Extinction co-efficient
µl	:	Microliter
µm	:	Micrometer
µM	:	Micromolar
3D	:	Three-dimensional
A	:	Absorbance
aa	:	Amino acid
ACC	:	1-Aminocyclopropane-1-carboxylate
ACO	:	Aminocyclopropane-1-carboxylate oxidase
ANOVA	:	Analysis of variance
ARF	:	Auxin response factors
arl1	:	Adventitious rootless1
AuxREs	:	Auxin responsive elements
bp	:	Base pair
bPDS	:	Barley phytoenedesaturase
BSA	:	Bovine serum albumin
BSMV	:	Barley stripe mosaic virus
C	:	Carbon
$Ca(NO_3)_2.4H_2O$	:	Calcium Nitrate Tetrahydrate
$CaCl_2.2H_2O$	:	Calcium chloride dihydrate
CASP	:	Critical Assessment of Structure Prediction
CbLCV	:	Cabbage leaf curl virus
CCSHAU	:	Chaudhary Charan Singh Haryana Agricultural University
CDD	:	Conserved Domain Database
cDNA	:	Complementary DNA
cm	:	Centimeter
COR1	:	Coronatine insensitive1
crl1	:	Crown rootless1
CTAB	:	Cetyl trimethyl ammonium bromide

$CuSO_4.5H_2O$	:	Copper(II) Sulfate Pentahydrate
d.f	:	Dilution factor
DAS	:	Day after sowing
DEPC	:	Diethylpyrocarbonate
DMC1	:	Disrupted meiosis cDNA1
DNA	:	Deoxyribose nucleic acid
DNase	:	Deoxyribonuclease
dNTPs	:	Deoxy nucleotide tri-phosphates
dpi	:	Days post inoculation
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
dsRNA	:	Double stranded RNA
DTT	:	Dithiothritol
e.g.	:	For example
EDTA	:	Ethylenediaminetetracetic acid
EGTA	:	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EST	:	Expressed sequence tags
et al.	:	et alia
F	:	Forward
$FeSO_4.7H_20$	:	Iron(II) Sulfate Heptahydrate
g	:	Gram
GLU3	:	Endo-1,4-b-glucanase
GRRs	:	Gene-rich regions
H	:	Hydrogen
h	:	Hour
H_2MoO_4	:	Molybdic acid
H_2O_2	:	Hydrogen peroxide
H_3BO_3	:	Boric acid
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercury(II) chloride
IAA	:	Indole-3 acetic acid
IBM	:	International Business Machinery
IITR	:	Indian Institute of Technology Roorkee, Roorkee

IPP	:	1-[6-chloro-3- methyl-pyridyl-8-nitro-7-methyl-1 2 3 5 6 7-hexahydro imidazo-(1,2a)]-pyridine
iTASSER	:	Iterative Threading ASSEmbly Refinement
Kb	:	Kilo bases
KCl	:	Potassium chloride
KH_2PO_4	:	Monopotassium phosphate
KNO_3	:	Potassium nitrate
L	:	Litre
LB	:	Luria broth
LOB	:	Lateral organ boundaries
m	:	Meter
M	:	Molar
MBCF	:	Molecular Biology Core Facilities
MCS	:	Multiple cloning site
MEGA	:	Molecular Evolutionary Genetics Analysis
mg	:	Milligram
$MgCl_2$	:	Magnesium chloride
$MgSO_4.7H_2O$	:	Magnesium Sulfate Heptahydrate
min	:	Minute
ml	:	Millilitre
mm	:	Millimeter
mM	:	Millimolar
$MnCl_2.4H_2O$	:	Manganese(II) Chloride Tetrahydrate
mRNA	:	Messenger RNA
N	:	Nitrogen
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
NCBI	:	National Center for Biotechnology Information
ng	:	Nanogram
nm	:	Nanometer
nt	:	Nucleotide
NTRA	:	NADPH-dependent thioredoxin reductases
NY	:	New York
O	:	Oxygen

OD	:	Optical density
PBS	:	Phosphate-buffered saline
PCNA	:	Proliferating cell nuclear antigen
PCR	:	Polymerase chain reaction
PDS	:	Phytoene desaturase
pH	:	Potential of hydrogen
PIPES	:	Piperazine-N, N′-bis(2-ethanesulfonic acid)
pM	:	Picomolar
POD	:	Peroxidase
PTGS	:	Post transcriptional gene silencing
p-value	:	Calculated probability
PVP	:	Polyvinylpyrrolidone
PVX	:	Potato virus X
QTLs	:	Quantitative trait loci
R	:	Reverse
RAPD	:	Random amplified polymorphic DNA
RAPDB	:	Rice Annotation Project Database
RGCs	:	Resistance gene candidates
RH	:	Radiation hybrid
RNA	:	Ribose nucleic acid
RNaseA	:	RibonucleaseA
RNaseH	:	RibonucleaseH
ROS	:	Reactive oxygen species
rpm	:	Revolutions per minute
rth 1	:	Roothairless1
SAVES	:	Structural Analysis and Verification Server
SD	:	Standard deviation
sec	:	Second
siRNAs	:	Small interfering RNAs
SOC	:	Super Optimal Broth
SOD	:	Superoxide dismutase
SPSS	:	Statistical Package for the Social Sciences
TAIR	:	The Arabidopsis Information Resource
Taq	:	*Thermus aquaticus*

TBE	:	Tris borate EDTA
tBLAST	:	Translated Basic Local Alignment Search Tool
$TE/T_{10}E_1$	:	Tris EDTA
TGMV	:	Tomato golden mosaic DNA virus
TRV	:	Tobacco rattle virus
U	:	Unit
UGMS	:	Unigene derived microsatellite
USA	:	United States of America
UV	:	Ultraviolet
V	:	Volume
VIGS	:	Virus-induced gene silencing
WSU	:	Washington State University
$ZnSO_4.7H_2O$	:	Zinc sulfate heptahydrate
α-Man	:	α-mannosidase
β-Hex	:	β-D-N-acetylhexosaminidase

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Chapter 1

Introduction

1. INTRODUCTION

Wheat (*Triticum* spp.) is one of the major staple food crops of the world in terms of food source and cultivated area. Wheat alone contributes ~28 % of world's edible dry matter and up to 80 % of daily food intake in several developing nations [10, 58]. Wheat belongs to the tribe Triticeae in the grass family Poaceae that also contains major crops like rice (*Oryza sativa* L.), maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.). The hexaploid wheat, *Triticum aestivum* (2n(6x)=42) has three different genomes designated as A, B and D. Two separate natural amphiploidization events occurred during the evolution of allohexaploid wheat [201]. Diploid einkorn types of wheat were the oldest while the hexaploid wheat (*T. aestivum*) is recently evolved [91].

A number of efforts have been made to improve wheat varieties for effective grain yield, biological yield and number of grains per ear in last two decades [97, 187, 196, 206, 207]. Nutrient tolerance is considered as one of the important factors that affect the grain yield in wheat [46]. Nitrate, however, is the most common form of nitrogen taken up by cereal crops in the field. Localized supply of nitrate is considered as one of the factors that are responsible for the elongation of lateral roots [251]. The activation of nitrate absorption by plants is under genetic control [157] and considerable differences exist among cultivars. The variation in nitrate uptake and transport in wheat is due to the differences in size and morphology of the roots, and the demand for mineral elements at different growth stages [68].

One of the modern approaches to decipher the function of various genes is named as gene silencing. Gene silencing in general refers to the ability of a cell to prevent the expression of certain genes at transcriptional or translational level. The history of gene silencing came into existence in late eighties when some researchers found that only modifications of DNA or protein could lead to transcriptional repression or activation [132]. Later, the concept of post-transcriptional gene silencing was introduced on the basis of a number of gene-silencing phenomena that were discovered in plants, fungi, animals and ciliates [14, 152]. Post-transcriptional gene silencing against a wide range of viruses, also known virus induced gene silencing (VIGS), is also utilized as a natural defense method in plants. VIGS has been extensively used to study the function of unknown genes in various plant species [13, 15, 198]

Plant root architecture that includes root length, root density and number of lateral roots is a critical agronomic trait determining the environmental stress adaptability and productivity in crops[44]. The monocot cereals have a complex root structure comprising of primary embryonic roots and post embryonic crown roots. The development of primary embryonic roots is followed by the development of crown roots which when fully developed constitute the major part of the mature plant root system providing lodging resistance, mineral uptake and water uptake capacity to the plant [69, 232, 251].

Molecular genetic studies on the development of crown roots have been done in maize and rice. Thirteen genes, viz., *CAND1, COW1, CRL1, CRL2, CRL3, GNOM1, MT2b, CRL5, SHR1, SCR1, SCR2, WOX11, YUCCA1* involved in crown root formation have been identified in rice [240]. The rice mutant defective in the *CRL1* gene lacks crown root initiation[137]. The *CRL1* gene mutants of rice had fewer lateral roots and altered root gravitropism, though these mutants had normal embryonic roots [101]. A maize mutant impaired in the rootless concerning crown and seminal roots (*RTCS*) gene, an ortholog of *CRL1* gene of rice, was found to be impaired in the initiation of embryonic seminal roots and post-embryonic shoot-borne root system [214]. Majer and Hochholdinger (2011) reported that lateral organ boundary domain (LBD/LOB domain) of *RTCS* gene is responsible for defining organ boundaries and development of almost every part of the maize plant [145]. The LOB domain was found to consist of C-motif required for DNA binding [204] and leucine-zipper like motif responsible for protein-protein interactions [150]. An ortholog of *CRL1*/*RTCS* gene has not yet been identified in wheat.

The evolutionary and structural studies in wheat with different genes have been done by many workers [4, 54, 114, 165, 181]. These workers compared the gene structure and protein conservation of different genes among dicots and monocots. No such study has been done in *RTCS* gene so far. Hence, the present study was done to identify and functionally characterize *RTCS* gene of wheat using bioinformatic and virus induced gene silencing (VIGS) approaches. The effect of nitrate deficiency on root architecture was also studied in eight wheat varieties.

The current research work was carried out with the following major objectives:

1) Biochemical and molecular studies on root architecture in eight wheat varieties in response to nitrate stress
2) Identification and characterization of wheat *RTCS* gene
3) Preparation of VIGS construct and infection of wheat plants with the prepared construct
4) Phenotypic and molecular characterization of the silenced wheat plant

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

The literature on the root architecture modification and related aspects has been reviewed below under suitable headings.

2.1. Wheat genomics research

The research work on genomics of wheat has been recently reviewed by two groups [82, 125]. Arumuganathan and Earle [9] found the nuclear content of wheat as 16 billion bp per haploid genome using flow cytometry method. Gill and coworkers [75] observed the distribution of genes and recombination in group 5 chromosomes of wheat by comparing genetic and physical maps. These workers found that more than 60 % of the long arm markers were present in 3 major clusters encompassing less than 18 % of the chromosomal arm. Similarly, by comparing the high-density physical and genetic maps, the distribution of genes and recombination in group 1 chromosomes of wheat was also studied by Gill and coworkers [76]. These workers found that 86 % of the group 1 markers were present in five clusters that encompassed only 10% of the wheat group 1 chromosome. Similar findings were observed by Gustafson and coworkers [84]. Erayman *et al.* [67] mapped 3025 loci and found that 29% the wheat genome comprises of 18 major and 30 minor gene-rich regions (GRRs). Recombination was found to occur mainly in these GRRs. Qi and coworkers [183] mapped 7,104 expressed sequence tags (ESTs) loci in wheat. More loci were mapped in the B genome (5,774) than in the A (5,173) or D (5,146) genomes. They found that density of EST in D genome was significantly higher than for the A or B. It was observed that most of the agronomically important genes were positioned in the dense EST regions.

Molecular markers have been extensively studied in various crop species [16, 41, 78, 83, 113]. Molecular markers are important system for studying polymorphism in crops. Myburg and coworkers [161] used randomly amplified polymorphic DNA (RAPD) fingerprinting profile to distinguish different wheat cultivars. Devos and coworkers [52] converted microsatellite sequences into PCR-based markers. These markers were highly specific and displayed a high level of variation in wheat. Johal and coworkers [108] introduced a new approach i.e mutant assisted gene identification and characterization (MAGIC) in crops for identifying novel genes and variants for a trait. Recently, Wang and coworkers [228] characterized wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. These workers mapped a total of 46 977 SNPs using a combination of eight mapping populations.

2.2. Physical mapping in wheat

Endo [65] observed the induction of chromosomal structural changes by a chromosome of *Aegilops cylindrica* (progenitor) in wheat. Werner and coworkers [234] observed numerous deletions in the progeny of a monosomic addition of a chromosome from *Triticum cylindricum* in wheat, due to single breakpoints and a concomitant loss of distal fragments. These researchers constructed physical maps by using 41 deletions from chromosomes 7A, 7B and 7D, and a set of genetically mapped DNA probes. Gill and coworkers [74] described a method for region-specific mapping in hexaploid wheat. These workers utilized 26 deletion lines to allocate the markers to specific chromosome regions by deletion mapping. More than 400 chromosome deletion lines for all of the 21 chromosomes in wheat were reported by Endo and Gill [66]. Mickelson-Young *et al.* [154] reported the mapping of homoeologous chromosomes 4A, 4B, and 4D using 39 homozygous deletion lines in wheat. Weng and coworkers [233] showed the extended physical map of group 6 chromosome of wheat using deletion lines.

Erayman *et al.* [67] reported the physical mapping of 3025 loci including 252 phenotypically characterized genes and 17 quantitative trait loci (QTLs). These loci were relative to 334 deletion breakpoints. Maleki and coworkers [147] mapped 137 resistance gene like sequences, kinase analogs and NBS-LRR resistance gene analogs. These were amplified both physically and genetically from genomic DNA on 20 wheat chromosomes. Dilbirligi *et al.* (2004) [56] reported the physical mapping of 121 expressed resistance gene candidates (RGCs) using 339 deletion lines. They observed five major *R*-gene clusters that spanned only 3 % of the wheat genome but contained 47 % of the candidate *R* gene. Conley *et al.* [45] constructed a high-density EST chromosome bin map of wheat homoeologous group 2 chromosomes using deletion stocks. These workers mapped 2600 loci generated from 1110 ESTs to the group 2 chromosomes by southern hybridization. In another study, Randhawa and coworkers [186] identified 882 ESTs from homoeologous group 6 of wheat by physically mapping 7965 singletons from 37 cDNA libraries using aneuploid and deletion stocks.

Apart from deletion mapping, *in-silico* physical mapping is also one of the methods to construct molecular markers based physical maps. Markers with known sequences can be mapped to wheat chromosomes through sequence similarity with mapped EST loci available at GrainGene database (http://wheat.pw.usda.gov/GG2/blast.shtml [151]. Parida *et al.* [177] used *in-silico* method to find the unigene derived microsatellite (UGMS) markers in five cereal species

namely, wheat, rice, barley, maize and *sorghum*. Mohan and coworkers [158] utilized both *in-silico* and wet lab based approaches to map 672 EST loci.

Kalavacharla *et al.* [110] constructed high-resolution radiation hybrid (RH) map of chromosome 1D in wheat. These workers reported an RH panel of 87 lines to map 378 molecular markers that detected 2312 chromosome breaks. Using similar approach, 57 markers were used to make a map of 205.2 cR (centiRay) in bread wheat by Michalak De Jimenez *et al.* [48]. Recently, Luo and coworkers [143] had constructed a 4-gigabase physical map unlocking the structure and evolution of the genome of *Aegilops tauschii,* the wheat D-genome progenitor. These investigators have fingerprinted 4,61,706 bacterial artificial chromosome clones with the aim to map genes and to find the gene-rich regions (GRRs).

2.3. Root architecture in plants

The research work on root architecture in plants has been recently reviewed by Smith and De Smet [209]. Primary roots derived from embryonically formed meristematic tissue are the first roots to emerge in both monocots and dicots; however, adventitious (crown) roots are formed post-embryonically from root-shoot junction that is a feature of monocot only. Root morphology in both monocot and dicot plants indicates the presence of vascular bundles inside a central vascular column and stele surrounded by epidermal, cortical and endodermal cells, as shown in Fig. 2.1. [209].

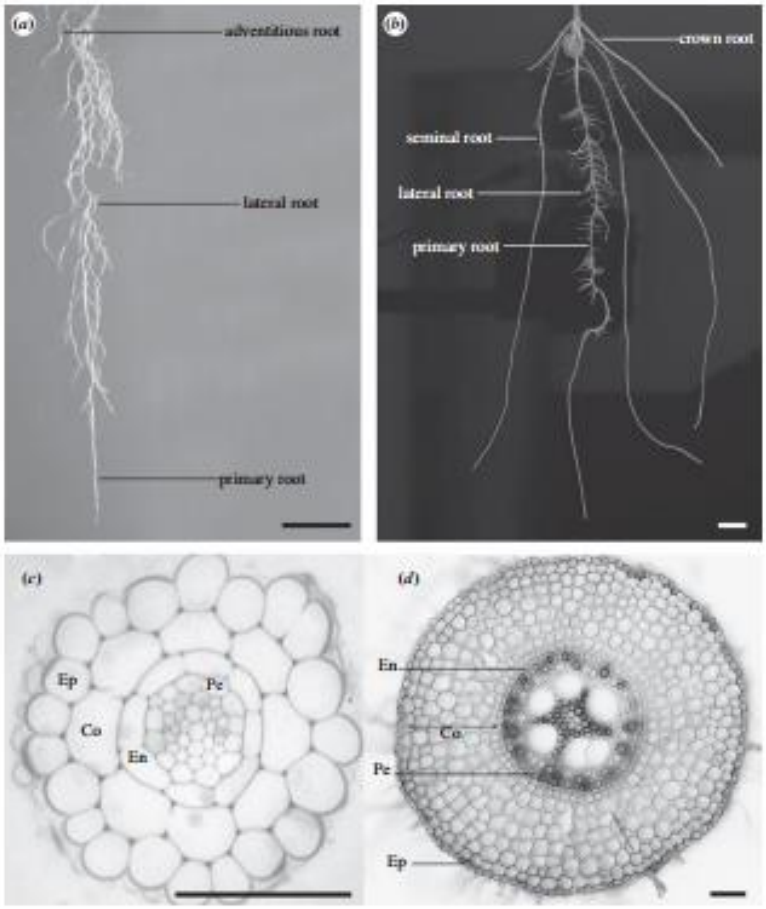

Fig. 2.1. Root system of *Arabidopsis* and Maize a) Major root types of 14 day old *Arabidopsis* and b) 10 day old maize c) Transverse section through *Arabidopsis* and d) maize root. The endodermis (En), cortex (Co), pericycle (Pe) and epidermis (Ep) are indicated. Permission granted from Royal Society of Biological Science.

Dolan and coworkers [59] described the anatomy of developing root in *Arabidopsis* using conventional histological techniques, transmission and scanning electron microscopy. They found that the organization of cells is similar in lateral roots and primary root but more variable in the numbers of cell files in each layer. Dubrovsky *et al* [61] studied the early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana*. They reported that pericycle cells in direct contact with underlying protoxylem cells participate in lateral root primordia (LRP) formation. Auxin dependent cell cycle progression is the primary reason for lateral root initiation in pericycle cells [92]. De Smet and coworkers [50] characterized the receptor –like kinase *ACR4* gene responsible for lateral root initiation and formative cell divisions in the pericycle of

Arabidopsis. *ACR4* function revealed a common mechanism of formative cell division control in the main root tip meristem and during lateral root initiation.

Monocots including maize and rice bear a complex root system as compared to the dicots. Roots of monocots are larger, and their primary roots consist of 10-15 cortical layer of cells while dicots possesses single layer [195]. Jiang and coworkers reported the presence of 800-1200 cells in the quiescent centre (found inside root apical meristem) in maize [107]. These cells were comparatively more than that of *Arabidopsis* which has only 4 cells present in the quiescent centre [59].

Apart from primary and lateral roots, monocots and cereals consist of fibrous root system (brace and seminal) having different types of branched root, thus making their overall root architecture more complex. Hochholdinger and coworkers [95] observed the emergence of crown roots from stem nodes (below- ground) after 5-10 days of germination, whereas brace roots emerged from stem nodes (above-ground) after 6 weeks of germination in maize. As compared to dicots where lateral root formation begins from xylem poles, in monocots, this process begins at phloem pole [29]. Only 2 xylem poles were found responsible for the initiation of lateral roots in *Arabidopsis* [60], whereas, ten phloem poles were also found responsible for initiating lateral roots [94].

2.4. Factors affecting root development in plants

Recently, the research work on the factors affecting root architecture development in plants has been has been reviewed by Jung *et al.* [109]. The development in roots is dependent on various intrinsic factors (hormones, receptors, transcription and signaling components) and extrinsic factors (environmental stimuli and their downstream signal transduction).

2.4.1. Effect of hormones on roots

Nakamura and coworkers [162] identified an auxin responsive gene *IAA3* in rice which was responsible for abnormal shoot and root gravitropism, auxin insensitivity and abnormality in lateral root initiation. Another auxin responsive gene, *PIN1*, found in *Arabidopsis* and rice was responsible for tillering and root emergence [242]. Alterations in adventitious root number were found due to the over expression of *YUCCA 1* gene in rice [243]. Wang *et al.* [226] reported that auxin responsive factors, *ARF10* and *ARF16*, were involved in the root cap cell formation of primary roots and lateral roots in *Arabidopsis*. Two studies showed that the development of the

crown roots and root meristem was affected due to the alteration in the expression of *WOX11* gene [255] and *MT2b* gene [247] in rice.

2.4.2. Effect of environmental factors on roots

The effect of nitrate as a fertilizer on growth and nodulation in plants have been studied by many workers [77, 176]. Nitrogen in the form of nitrate is taken up by plants using three transport systems, namely, high affinity transport system (HATS), inducible high affinity transport system (iHATS) and low affinity transporter system (LATS) [119]. Williams and Miller [236] studied the uptake of ammonium in *Arabidopsis*. These workers revealed that both ammonia and nitrate were responsible for increasing the root and shoot biomass yield. Rasmussen and coworkers reported that damaged roots of wheat plants could uptake protein directly from the soil and transport it to stem. The molecular mechanism of this phenomenon is still unknown [188].

Tsay *et al.*[218] described the function of *NRT 1.1* gene as a soil nitrate concentration sensor and auxin uptake facilitator. These workers hypothesized that lateral root elongation was due to the effect of low soil nitrate concentration. In *Arabidopsis*, more than 50 *NRT1* genes (low affinity transporters) [236] and 7 *NRT2* genes (high affinity transporters) [175] have been reported. Apart from nitrate transporter genes, other genes such as *NAR* [244], *NR1* [133], *GS1* [212] and *NRR* [254] have been found responsible for altering the root growth in response to nitrogen availability in rice.

Another micronutrient that is responsible for elongation of roots is phosphorus. Wang and coworkers [229] described that the changes in root epidermis, lateral roots and root hairs were due to the alterations in the expression of phosphorus transporter genes, *OsPT9* and *OsPT10*, in rice. The alteration in expression was found to be induced by phosphate starvation. Zhou *et al.* [258] reported that over expression of *PHR2* gene under low phosphate conditions resulted in proliferation of root growth in rice.

Apart from nutrients, root architecture in plants is also affected by water availability. Singh *et al.* [208] reported that *Sorghum* possessed vertically oriented root structure in response to drought. Under mild salinity conditions, inhibition of primary root elongation and promotion of lateral root emergence in *Arabidopsis* was observed by Zolla and coworkers [259]. Two

studies have shown that inoculation with salinity tolerant *Azotobacter* strains in saline soils increased the biomass and yield production in wheat plants [33, 164].

2.5. Defense mechanisms in plants against stresses

In plants, reactive oxygen species (ROS) produced by various metabolic pathways occurring inside organelles such as mitochondria, peroxisomes and chloroplast has been discussed in detail by two groups [142] [166]. Plants maintain their redox homeostasis by scavenging these ROS molecules by several defense mechanisms. The research work published on this aspect has been reviewed by Foyer and Noctor [71]. Chen and Dickman [34] reported that proline is a potent non-enzymatic antioxidant that effectively scavenge ROS and protect the cells from lethal stresses and prevented ROS mediated cell death.

Rana and coworkers [185] studied the effect of plant growth promoting rhizobacteria in eliciting the production of defense enzymes in wheat under field conditions. Bhatt and coworkers [18] found ascorbate peroxidase: superoxide dismutase ratio to be a critical factor governing the stress tolerance potential of different varieties of finger millet. Moreover, Chen and coworkers [35] reported that on subjecting the wheat hybrid to high light stress (1500 μmol m^{-2} s^{-1}), their antioxidative defense system and xanthophyll cycle gets enhanced, which was responsible for their tolerance to photoinhibition.

Recently, Cha and coworkers [31] reported the involvement of cytoplasmic NADPH-dependent thioredoxin reductases (NTRA) in the plant stress response counteracting oxidative and drought stresses by regulating the amounts of ROS. Further, Zhang and coworkers [253] demonstrated that a significant changes in phosphorylation level (SCPL) of phosphoproteins took place under drought stress. These phosphoproteins were involved in the biological processes such as stress/detoxification/defense, RNA transcription/processing and signal transduction in wheat. Moreover, Wu and coworkers [239] studied the role of molybdenum in drought tolerance. These workers showed that molybdenum application could enhance the antioxidative defense system of the plant, improve the water utilization capacity and increase the osmotic-adjustment ability in winter wheat under drought stress.

The effect of insecticides (omethoate) induced stress on the wheat seedling was studied by Zhang and coworkers [250]. The study revealed that the activity of antioxidant defense system of the plant played major role in response against oxidative stress, which was the main cause of

reduced growth in wheat exposed to higher concentrations of omethoate. Similarly, Wang and coworkers [227] studied the effect of pesticides (1-[6-chloro-3- methyl-pyridyl-8-nitro-7-methyl-1 2 3 5 6 7-hexahydro imidazo-(1,2a)]-pyridine (IPP)) on the antioxidant defense system of the wheat plant.

Sharma *et al.* [200] observed a close relationship between the changes in growth and antioxidant enzymes activity after the exposure to Hi-foliar nutrient foliar spray at different growth stages in *Brassica juncea* under field conditions. The production of antioxidant enzymes was found to be dependent on the conditions given to plants as observed by Tiwari and coworkers [217]. These workers found that the level of antioxidant activity in *Carica papaya* L. (var. Pusa Dwarf) was more when plants were grown *in vitro* conditions than *in vivo*.

2.6. Molecular control of roots in crops

Molecular control mechanisms operate at different levels of root development including root elongation, crown root formation, root hair development and root thickness. Defective cell elongation resulting in shorter root length was found in the mutant of *SRT1* gene in rice by Ichii and Ishikawa [149]. Inukai *et al.* [100] found that mutants of *RRL1* and *RRL2* genes showed shorter roots and inhibition in the maintenance of cell elongation and root apical meristem and hence are involved in regulation of root elongation in rice.

Chhun and coworkers [37] reported that the mutant of *Lrt1* gene in rice showed impairment in lateral roots and reduced root gravity response due to altered auxin activity. In another study by same researchers, auxin responsive pathway genes, *Arm1* and *Arm2*, were found to be involved in lateral root formation in rice [38]. These workers observed that rice mutant of *arm1* gene showed defect in lateral root initiation, reduced root diameter and xylem development in roots whereas slight increase was observed in seminal root elongation. Similar functions were observed in case of *arm2* mutant except enhanced plant height, slightly reduced lateral root formation and impaired xylem development in roots than that of *arm1* gene.

Liu and coworkers[137] found a gene, adventitious rootless1 *(arl1),* controlling the initiation of adventitious root primordia in rice. This gene was found to express in lateral and adventitious root primordia, young pedicels, vascular tissues, scutellum, and tiller primordia. The crown rootless1 (*crl1*) mutant was found defective in the crown root formation in rice by Inukai *et al*. [101]. These workers further found that the expression of *Crl1* gene is directly regulated by

an auxin responsive factor in the auxin signaling pathway. Another gene, *CRL3*, involved in initiation and growth of adventitious roots in rice was identified by Kitomi and coworkers [120]. The same scientists also reported another gene, *GNOM1*(*CRL4*), in rice that was involved in the development of vascular tissues, lateral roots, adventitious root primordia, anthers, leaves, lemma veins and root tips [121]. This gene belongs to the family of *PIN* genes, where regulation of adventitious root development is mediated by polar auxin transport [139].

Mutants of roothairless1 (*rth 1*) gene in rice displayed strong reduction of root hairs and defect in plant growth [248]. Ding and coworkers [57] reported a gene, *RHL1*, that was involved in root hair development of plants. These workers observed that the ortholog of *Arabidopsis RHL1* gene, *OsRHL1*, belongs to subfamily C of the bHLH family in rice. Won and coworkers [238] discovered a root hair specific *EXPB* gene in rice that encodes the proteins involved in modification of cell wall during root hair morphogenesis. Similar function was observed for *OsEXPA17* gene expression by ZhiMing *et al*. [256]. These researchers found a point mutation at position 104 (Gly to Arg) of OsEXPA17 protein sequence was responsible for shortening of root hairs.

Zhang and coworkers [252] studied the function of endo-1,4-b-glucanase (*GLU3*) gene in rice. These workers found that *OsGLU3* gene is expressed in various tissues with strong expression in root tip, lateral root, and crown root primodia. During phosphate starvation, *OsGLU3* gene was found to target the cellulose synthesis in the cell wall of roots thus modulating the root elongation in plant. Qin and coworkers [184] identified a mutant of *OsDGL1* gene, an ortholog of *Arabidopsis DGL1*, that was responsible for smaller root meristem, shorter root cell length, alteration of matrix polysaccharides in root cell wall and finally death of root cells. Another gene, *WOX3A*, involved in organ development such as palea and lemma morphogenesis in spikelets, vasculature patterning and lateral-axis outgrowth in leaves, the numbers of lateral roots and tillers was found in rice by Cho and coworkers [40]. This gene is also involved in the root hair formation.

2.7. Rootless concerning crown and seminal roots (*RTCS*) gene and its function

The root system of maize is very complex comprising of lateral seminal, crown and brace roots. In maize, isolation and characterization of a mutant of *RTCS* gene, deficient in formation of

nodal roots, was identified by Hetz and coworkers [90]. These workers found that *RTCS1* and *RTCS2* are two allelic root-deficient mutants that are involved in complete lack of formation of crown and lateral seminal roots in maize. But *rtcs* mutants could still produce an embryonic primary root with lateral roots.

Hochholdinger *et al*. [93] found that cyclin expression is completely suppressed at the site of crown root formation in the nodal region of the maize root mutant *RTCS*. The *RTCS* locus was mapped to the short arm of chromosome 1 with the help of a co-segregating RAPD marker [90]. However, Krebs and coworkers [123] performed extended mapping for chromosome 1 of maize and found that *rtcs* is located 10 cM from bmc1014 and 3 cM from bmc1083 marker positions.

An orthologue for *RTCS* gene was characterized in rice as *CRL1* by Inukai *et al*. [101]. These researchers found that mutant of this gene shows impaired adventitious roots. The map-based cloning of the *RTCS* gene encoding an 25.5 kDa LOB domain protein located on chromosome 1S of maize was reported by Taramino and coworkers [214]. These workers observed that *RTCS* expression is induced by auxin. A paralogue gene, RTCL, for *RTCS* gene has been characterized in maize that showed 72 % identity towards LOB domain [214]. LBD plays a pivotal role in defining organ boundaries and development of almost every part of plant including embryo, roots, shoots, leaves, inflorescence, plant regeneration and pollen development [204]. Mutation in LOB domain causing asymmetric leaf has also been reported in A*rabidopsis* by Iwakawa *et al*. [104]. Majer *et al*. [146] found that *RTCS* regulates ARF expression via binding to LBD motifs (5'-GCGGCG-3') in the promoter of ARF genes, which suggests a regulatory feedback loop. Auxin response factors (ARF) bind directly to Auxin Responsive Elements (AuxREs) of the *RTCS* promoter in vitro. These researchers also found that *RTCS* gene was located in the nucleus while its paralogue RTCS-LIKE (*RTCL*) was located in the nucleus and cytoplasm probably owing to an amino acid exchange in a nuclear localization signal. It was found that auxin response factor 34 can bind to the promoters of both the genes and activates their expression.

2.8. History of RNAi

Napoli and coworkers [163] tried to obtain transgenic petunias with greater amounts of anthocyanin pigments, by amplifying the gene activity of chalcone synthase. However, white or

chimeric flowers were produced instead of deep purple ones. It was found that the transgene was not expressed and rather ended up in the suppression of a homologue endogenous gene. Similar results were observed in the fungus *Neurospora crassa*, when Cogoni *et al* [42] studied the silencing effect of albino-1 (*al-1*), gene involved in carotenoid production. They found that at least 132 bp of the sequence homologous to the transcribed region of the native gene is required to induce the silencing. They coined this process as quelling. Elmayan and Vaucheret [64] introduced a bacterial *uidA* into tobacco plants and found the timing of silencing occurred at different rates in the different transformants. They concluded that post transcriptional gene silencing (PTGS) can occurs through a dose effect and not through DNA-DNA interaction.

The first report on gene silencing in animals was given by Guo and Kemphues in *C. elegans* [80]. They reported the function of *par-1* gene, which encodes a serine threonine kinase responsible for cleavage of developing embryo using antisense *par-1*. Earlier these researchers hypothesized that only the antisense gene hybridizes with endogenous mRNA that resulted in a double stranded RNA (dsRNA). Thus, either translation would have inhibited or degradation of the dsRNA complex could have been done by cellular ribonucleases. However, the similar silencing results were found using the sense *par-1* RNA by Guo and Kemphuses.

This puzzle of all the previously reported silencing of genes by cosuppresion, quelling, sense and antisense mRNA was solved by Fire *et al.* [70]. They were also working with *C. elegans* and found that the reason for gene silencing was dsRNA and not the single stranded RNA. They purified the sense and antisense ssRNAs and compared them with dsRNA. Surprisingly, they found that the dsRNA caused potent and specific interference on injected animals than sense or antisense ssRNA separately. They coined the term RNAi for this process.

Pruss and coworkers [182] revealed that potyviral P1/HC-Pro region affects a step in disease development which, is common to a broad range of virus infections and suggested a mechanism involving transactivation of viral replication. Ratcliff and coworkers observed that the post-transcriptional gene silencing of nuclear genes is a manifestation of a natural defense mechanism that is induced by a wide range of plant viruses [190]. Gene silencing in tomato was reported by Hamilton and Baulcombe [86]. These researchers identified a class of small RNAs of about 25 nucleotide (nt) as the triggering signal for silencing of aminocyclopropane-1-carboxylate oxidase (*ACO*) gene in tomato.

2.8.1. RNAi pathway

The research work on mechanism on RNAi has been reviewed by Hannon [89]. Kennerdell and Carthew [115] found that injecting of dsRNA into *Drosophila* embryos induces sequence-specific silencing and thus these embryo extracts becomes a competent model for studying the silencing mechanism. It was observed that incubating the dsRNA in these cell-free lysates reduced their ability to synthesize luciferase from a synthetic mRNA. This was further correlated with the observations made by Tuschl *et al.* [220]. These workers suggested that dsRNA might result in silencing by triggering the assembly of a nuclease complex that aims homologous RNAs for degradation. This nuclease which was isolated from the *Drosophila* S2 cells incubated with dsRNA is now known as RISC (RNA-induced silencing complex) [87]. Zamora *et al.* [249] found that small RNAs (21-23 nt) were produced from dsRNAs in *Drosophila* embryo extracts when these extracts were injected with dsRNAs. Further, partial purification of the RISC complex showed that these small RNAs consist of nuclease activity [87]. Thus, it was hypothesized that initiation of silencing occurs upon recognition of dsRNA by a machinery that converts the silencing trigger to ~21–25-nucleotide RNAs. These small interfering RNAs (siRNAs) are the key molecules of this family of silencing pathways and, by assembling with an effector complex RISC, they guide that complex to homologous substrates. The steps involved in RNAi has been described below.

2.8.1.1. The initiation step

Elbashir *et al.* [62] examined the structural determinants of short interfering RNAs including sequence, length, structure and chemical composition which are required to promote efficient RNAi. They found that siRNA duplexes of 21 nt with 2 nt 3' overhangs can trigger more specific mRNA degradation in a sequence specific manner. They also found that siRNA mediated degradation is sequence specific; mismatches prevent the target RNA cleavage. 5'end of guide siRNA determines the position of cleavage site at target RNA. Bernstein and coworkers [17] identified an enzyme of RNaseIII family nucleases named as Dicer. It consists of a helicase domain, dual RNaseIII motifs and a region of homology to RDE1/QDE2/ARGONAUTE family. They found that this enzyme works at initiation step and can produce putative guide RNA of appox. 22 nt. These guide RNAs further loaded over a nuclease complex known as RISC complex which degrades single stranded complementary mRNAs. Ketting *et al* [117] reported that mutations in *dcr-1* which is a homolog of Dicer in *C.elegans* hampers RNAi. Null mutations

in *dcr-1* gene leads to defective RNAi was found by Knight and Bass [122]. They suggested that the processing of dsRNA to short pieces is essential for effective RNAi. Blaszczyk, J. *et al.* [22] presented the crystal structure of RNase III from *Aquifex aeolicus.* They showed that RNase III consists of an N- terminal endonuclease domain complexed with Mg^{+} or Mn^{+} ions, a double stranded RNA binding domain and functions as a dimer. It also has 1-2 copies of a signature motif with consensus sequences of 9 residues.

2.8.1.2. The effector step

The effector step in RNAi involves a protein-RNA effector nuclease complex, known as RISC, which recognizes and destroys the target mRNAs. Hammond and coworkers [87] studied the mechanism underlying the RNAi silencing in *Drosophila.* They found that some nuclease activity in the transfected cells can degrade the dsRNA. They coined the term RISC for this enzyme and found that 25-nt RNA may confer specificity with this enzyme complex. The mechanism for RNA interference was further elucidated by Nykanen *et al* [171]. These workers found that RNAi requires 4 steps: 1) conversion of dsRNA into siRNAs with the help of ATP 2) incorporation of these siRNAs into a 360KD protein/ RNA complex 3) unwinding of siRNA duplex with the help of ATP to become active 4) recognition and cleavage of target RNA. Hammond and coworkers reported two steps which are involved in activation of RISC. First step involves changing the size and composition of subunit with the arrival of ATP. The second step describes the activation of RISC by AGO2- Argonaute gene family member [88]. The active fraction consists of a ribonucleoprotein complex of approximately 500 KDa.

These proteins belong to a very large evolutionarily conserved gene family with representatives in most eukaryotic genomes. They were initially referred as PAZ (Piwi Argonaut and Zwille) proteins due to the presence of a unique domain (PAZ) in their central part in the three members in which they are found: (a) Drosophila-P element-induced wimpy testis (Piwi), (b) Arabidopsis-argonaute1 (AGO1) and (c) Arabidopsis- zwille (ZLL) [30]. The Piwi domain of several PPD proteins was found to exhibit an RNaseH-like activity. Rivas and coworkers [192] reported that this domain is made up of a unique Asp-Asp-His (DDH) motif and performs endonuclease activity like RNaseH enzyme. These workers observed that Argonaute proteins catalyze mRNA cleavage within RISC effector complex. Irvine *et al* [102] showed that argonaute proteins require the conserved aspartate-aspartate-histidine motif for heterochromatic silencing and for ribonuclease H–like cleavage (slicing) of target messages complementary to

siRNA. It was concluded that siRNA guides histone modification by base-pairing interactions with RNA.

More than 20 related genes of Argonaute proteins have been linked to RNAi by genetic studies in *C. elegans*. Tabara *et al*. [211] isolated the two mutants of genes, *RDE-1* and *RDE-4*, resistant to RNAi. Later, the importance of *RDE-1* and *RDE-4* for initiation of silencing in a parental animal was reported by Grishok and colleagues [79]. It was found that both *RDE-1* and *RDE-4* were required for the formation of an interfering agent but were not needed for the interference thereafter. Parrish and Fire [179] observed that siRNA levels were greatly reduced in worms which were deprived of RDE-4 function, but these levels were found abundant in worms lacking *RDE-1*. These workers proposed a model in which *RDE-4* was involved before or during production of siRNAs, whereas *RDE-1* acts after the siRNAs have been formed. This study further revealed that mutations in the Argonaute family member *qde-2* in *Neurospora* eliminate quelling but do not alter the accumulation of siRNAs. These results suggested that *RDE-1* and other Argonaute proteins as well, might have a role in transportation of siRNAs to appropriate effector complexes (RISCs).

Apart from animals, few studies have been carried out in plants to understand the mechanisms of these Argonaut proteins. Vaucheret [222] carried out a phylogenetic comparison of 10 AGO proteins in *Arabidopsis* which classified these proteins in three major clades. Clade 1 contained AGO1, AGO5 and AGO10, clade 2 contained AGO2, AGO3 and AGO7, and clade 3 contained AGO4, AGO6, AGO8 and AGO9. This classification was based on protein sequence similarity and did not include functional similarity. Nonumura and coworkers [169] had divided the 18 AGO proteins in *Oryza sativa* into 4 clades in total among them the clade 1 was subdivided in two separate clades.

2.8.1.3. Amplification and spreading of silencing

Spreading of RNAi in the entire organism requires a method to pass a signal from cell to cell and an approach for amplifying that signal. Sijen and coworkers [205] explained that RNA directed RNA polymerase (RdRp) is responsible for the synthesis of secondary siRNA, from RNAi targeted mRNA. These secondary siRNAs, which were produced by the siRNA primed RdRp activity, consequently leads to RNAi amplification in *C. elegans*. Cogni and Macino [43] cloned quelling defective *qde-1* gene from *Neurospora crassa* and found that the product of this

gene is homologous to an RNA dependent RNA polymerase present in plants. This favors the conservation of RNAi mechanism among different species. Mourrain *et al.* [160] reported that *Arabidopsis* SGS 2 protein is similar to an RNA dependent RNA polymerase *QDE-1* of *N. crassa* and *EGO-1* of *C.elegans*, which controls quelling and RNAi respectively. They also suggested that PTGS, quelling and RNAi shared a common mechanism for target RNA degradation. Lipardi *et al.* [136] revealed that siRNA might be involved in the synthesis of long dsRNA.

2.8.2. Virus induced gene silencing (VIGS)

The research work on virus induced gene silencing in plants has been reviewed by Bhullar and Keller [20]. With the advent of RNAi, several workers reported the different mechanisms involved in the silencing.

A counter defensive strategy of plant viruses was studied by Kasschau and Carrington [112]. These researchers found that the P1/HC-Pro polyprotein encoded by tobacco etch virus acts as a suppressor of PTGS. One of the possible evolutions of PTGS can be an antiviral mechanism in plants, as described by Ratcliff and coworkers [190]. These workers found that the post-transcriptional gene silencing of nuclear genes is a manifestation of a natural defense mechanism that is induced by a wide range of plant viruses. Voinnet and coworkers [224] discovered three suppressors of PTGS and showed that suppression of PTGS is widely used as a counter-defense strategy by DNA and RNA viruses. Thus, this antiviral mechanism of plants as a PTGS gave a clue to develop a virus based technique for studying the gene silencing.

Van Kammen [221] first described the term 'virus-induced gene silencing' in plants as a spectacle of reclamation from virus infection. Thomas and coworkers [216] explored the role of length of RNA sequence in PTGS and found out that the lower size limit required for silencing was 23 nucleotides, which corresponds to exact size of small RNAs associated with PTGS in plants. Apart from the length of nucleotide sequence, other factors such as thermodynamic properties of the siRNA [118] and nucleotide configuration of the targeting sequence [216] also play important role in deciding the efficiency of silencing.

VIGS can be done by using many viral vectors. The first viral vector used in VIGS was a tobacco mosaic virus (TMV) by Kumagai and coworkers [124]. In this experiment, tobacco plants were inoculated with TMV carrying a phytoene synthase and PDS inserts, which resulted in photobleaching of tobacco leaves by degradation of its RNA. This provided a breakthrough in

the field of RNAi and a potential tool to decode the function of endogenous genes in plants. Peele *et al.* [180] reported extensive silencing of endogenous genes encoding proliferating cell nuclear antigen *(PCNA)* and a subunit of magnesium chelatase in tobacco using tomato golden mosaic DNA virus (TGMV). Ratcliff and coworkers [189] reported the development of vectors based on tobacco rattle virus (TRV). The TRV target host RNAs in the growing points of plants as compare to other RNA virus vector. Liu *et al.* [140] used TRV based VIGS approach to explore the role of tobacco candidate genes i.e. *Rar1*, *EDS1*, and *NPR1/NIM1* in the N-mediated resistance to TMV.

The earlier studies were restricted to dicots only. Holzberg and coworkers [96] demonstrated VIGS for the first time in monocot plant by using modified BSMV in barley. A modified BSMV was developed by Lacomme *et al.* [128] to get higher efficacy of silencing. This modified vector consisted of 40–60 base pair direct inverted repeats that produce dsRNA hairpins upon the transcription of the TMV. Later this modified BSMV vector was efficiently used in silencing of various genes in wheat including *lr21*, phytoene desaturase, *RAR1*, *SGT1*, and *HSP90* [198], seed-specific granule-bound starch synthase, disrupted meiosis cDNA1 (*DMC1*) and coronatine insensitive1 (*COR1*) [15].

Apart from RNA based vectors, Turnage *et al.* [219] developed a system based on bipartite geminivirus cabbage leaf curl virus (CbLCV) derived vector (a DNA based vector) for transient silencing of genes in *Arabidopsis*.

2.8.3. Application of RNAi in crop improvement

The research work on the use of RNAi to improve the quality of numerous plants has been reported in the review by Jagtap *et al.* [105]. The manipulation of pathways involved in RNAi can generate small RNAs that can further be used for producing quality traits and yield improvement in plants.

Liu and coworkers [138] modified the fatty acid composition of cottonseed oil by down regulating the expression of two key fatty acid desaturase genes, *ghFAD2-1*-encoding oleoyl-phosphatidylcholine omega 6-desaturase and *ghSAD-1*-encoding stearoyl-acyl-carrier protein Delta 9-desaturase using hpRNA mediated gene silencing. The silencing of *ghSAD-1* and *ghFAD-2* genes substantially enhanced the level of stearic acid and oleic acid, respectively. The level of palmitic acid was significantly reduced in both high-stearic and high-oleic lines [138].

Another application of RNAi was found by Ogita and coworkers [172] in producing decaffeinated coffee by down-regulating the expression of the gene encoding theobromine synthase (CaMXMT1), an enzyme involved in caffeine biosynthesis in coffee (*Coffea canephora*) plants.

Kusaba *et al.* [126] to reduced the glutelin content in rice grains by suppressing of expression of *Low glutelin content* 1 (*Lgc1*) gene. Amylose content was increased in wheat endosperm by Regina and coworkers [191], by suppressing the expression of starch-branching enzyme *SBEIIa* and *SBEIIb* genes. Moreover, Shimada and coworkers [203] produced sweet potatoes (*Ipomoea batatas*) with higher amylose content by following the same approach.

Davuluri *et al.* [47] enhanced the nutritional value of tomato (*Lycopersicon esculentum*) fruit by increasing their carotenoid and flavonoid content using RNAi technology. In this study, *DET1*, an endogenous photomorphogenesis regulatory gene was suppressed using a fruit specific promoter. Similarly in rapeseed (*Brassica napus*), the expression of lycopene epsilon cyclase (*ε-CYC*) gene was down-regulated using RNAi to increase the content of carotenoids namely violaxanthin, β-carotene, lutein and zeaxanthin in seeds of *B. napus* [246].

For increasing the bioavailability of minerals and micronutrients, a lot of work has been carried out to produce crops with low myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP6; phytic acid) phytic acid content. Shi and coworkers [202] produced low-phytic acid *lpa*2 mutant by mutating the maize inositol phosphate kinase (*ZmIpk*) gene using a reverse genetics approach. Kuwano *et al.* [127] reduced the phytic acid level in rice seeds by manipulating the expression of enzyme Ins (3) P1 synthase gene *RINO1* responsible for the phytic acid biosynthesis. In soybean (*Glycine max*), Nunes and coworkers [170] reduced the phytate content (94.5 %) by silencing the myo-inositol-1-phosphate (GmMIPS1) gene. This reduction of phytate content was correlated with seed development. Bowen *et al.* [24] developed low phytic acid M955 *lpa* mutant in barley (*Hordeum vulgare* L.) using RNAi.

Allen and coworkers [5] reported for the first time the gene silencing in transgenic opium poppy (*Papaver somniferum*) and silenced the codeine reductase (*COR*) by using chimeric hairpin RNA construct. These researchers revealed that the precursor alkaloid (S)-reticuline and their methylated derivatives (a non-narcotic alkaloid) gets accumulated in the transgenic plants at

the expense of narcotic alkaloids including oripavine, morphine, thebaine and codeine. Disruption of gossypol biosynthesis using RNAi in cottonseed was observed by Sunilkumar *et al.* [210]. These scientists studied that down-regulation of expression of δ-cadinene synthase gene resulted in the reduction of gossypol content during seed development.

Allergenicity and toxicity caused by various food allergens has been reduced using RNAi technology. Because of high sequence specificity, RNAi targets only the allergens without hampering the metabolites produced from plants. The expression of *Mal d 1* gene in apple is a major cause for apple allergy. Gilissen and coworkers [73] reduced the expression of Mal d 1 allergen in apple using RNAi mediated silencing. Profilin is an actin binding protein and major food allergen in fruits and vegetables including tomato. Le *et al.* [129] reduced the profilin accumulation in transgenic tomato by silencing the genes (i.e. *Lyc e 1.01* and *Lyc e 1.02*) responsible for encoding profilin in tomato by means of RNAi.

Houmard and coworkers [98] enhanced the accumulation of lysine in the maize kernel by down regulating the expression of lysine catabolic enzyme lysine-ketoglutarate reductase/saccharopine dehydrogenase (ZLKR/SDH) by using RNAi. Xiong *et al.* [241] produced transgenic tomato having an insert of 1-aminocyclopropane-1-carboxylate (*ACC*) oxidase dsRNA that hinder the synthesis of ACC oxidase, which is required to catalyzes the oxidation of ACC to ethylene, a plant growth regulator that plays an important role in ripening, thus, enhanced the self-life of tomato. Similarly, Meli and coworkers [153] enhanced the shelf life of tomato by suppression of two ripening-specific N-glycoprotein modifying enzymes namely α-mannosidase (α-Man) and β-D-N-acetylhexosaminidase (β-Hex).

The applications of RNAi along with their specific roles in various plants have been presented in Table 2.1.

Table 2.1. Application of RNAi in crop improvement (modified from Jagtap *et al.* [105])

Application	**Description**
Alteration of plant architecture	Adventitious root emergence and development were inhibited in the OsPIN1 RNA interference (RNAi) transgenic plants in rice [242]
	OsGLU1 gene down-regulation resulted in structural changes of cell wall in internodes of rice [257]
Abiotic stress tolerance	Transgenic rice showing silencing of *RACK 1* gene resulted in drought tolerance [106]
	Down-regulation of *UBC24* gene in *Arabidopsis* produced more abiotic stress tolerant lines [39]
Biotic stress resistance	Coat protein mediated transgenic lines were developed in papaya to get resistance against viral attacks [116]
	Transgenic tomato lines were produced resistance against potato spindle viroid using hpRNA constructs [197]
Development of male sterile plants	Down-regulation of anther-specific gene *TA29* in tobacco produced male sterile lines by RNAi[167]
	Transgenic sterile lines in rice were produced by suppressing the effect of OsGEN-L gene using RNAi [159]
Modulation of flower color and scent	The flower color of *Torenia hybrida* (a garden plant) was modulated from blue to white or pale color by down-regulating the chalcone synthase gene [72]
	Silencing of the phenylacetaldehyde synthase gene (*PhPAAS*) led to the elimination of the emission of phenylacetaldehyde and 2-phenylethanol in *Petunia* [111]
Seedless fruit development	Suppression of *ARF7* gene in tomato resulted in production of seedless fruit using RNAi approach [49]

It is evident from the above literature that plant root architecture is the critical agronomic trait which determines the environmental stress adaptability and productivity in crops. Despite the global importance of wheat, little information is available on its root architecture modification at molecular level in response to abiotic stress. An extensive research has been done in improving the plants using various molecular approaches like RNAi. However, this technique has not been utilized efficiently in cereal crops like wheat. Therefore, this work was planned to study the function of root architecture related genes in response to nitrate stress, and to identify and characterize the *RTCS* gene using VIGS approach in wheat.

Chapter 3

Materials and Methods

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant material

The seeds of eight wheat cultivars, namely, PBW 343, PBW 621, WL 711, WH 542, DBW 17, PBW 550, WH 1021 and WH 1025 were chosen for the nitrate stress experiment. The seeds of these cultivars were provided by Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), Hisar, India. The seeds of three wheat cultivars, viz., Chinese Spring, Lok 1 and HD 2967 were chosen for the virus induced gene silencing experiment. The seeds of these cultivars were kindly provided by Department of Crop and Soil Sciences, Washington State University, Pullman, USA with permission.

3.1.2. Chemicals/biochemicals and buffers used

All the chemicals/biochemicals used were procured from standard companies and were of analytical grade.

3.1.2.1. TE/$T_{10}E_1$ buffer

TE buffer was composed of 1 ml of 1 M Tris-HCl (pH 8.0) and 200 µl of 0.5 M EDTA dissolved in 100 ml MilliQ water.

3.1.2.2. TBE buffer (1X)

The composition of the TBE buffer was as follows:

Name of the reagent	**Composition**
Tris	10.9 g
Boric acid	5.56 g
EDTA (pH 8.0)	0.98 g
Distilled water	1 L

3.1.2.3. Preparation of DNA gel loading dye

The loading dye was prepared by mixing 0.25 % bromophenol blue and 0.25 % xylene cyanol in 50 % glycerol made in $T_{10}E_1$ buffer.

3.1.2.4. DNA extraction buffer

The composition of DNA extraction buffer was as follows:

Name of the reagent	**Concentration**
Tris-HCl (pH 8.0)	200 mM
EDTA (pH 8.0)	20 mM
NaCl	1.4 M
CTAB	10 %
β-mercaptoethanol	0.01 %

3.1.2.5. Polymerase chain reaction (PCR) mixture

The composition of PCR reaction mixture was as follows:

Contents	**Amount per 10 µl**
10X *Taq* buffer	1.0 µl
dNTP mix (10 mM) (Biotool, USA)	0.5 µl
$MgCl_2$ (50 mM)	0.5 µl
Taq polymerase (1 U/ µl) (Biotool, USA)	0.5 µl
Forward primer (20 pM)	0.5 µl
Reverse primer (20 pM)	0.5 µl
DNA (100 ng/µl)	1.0 µl
MilliQ water	5.5 µl

3.1.2.6. Master mix for PCR used in single stranded complementary DNA (cDNA) synthesis

The composition of PCR master mix reaction mixture was as follows

Contents	**Amount per 20 µl**
5X Reverse transcriptase buffer	4.0 µl
dNTP mix (10 mM)	0.5 µl
OligodT primer (20 pM)	0.5 µl
Reverse transcriptase (New England Biolabs) (10U/ µl)	0.3 µl
MilliQ water	4.7 µl

3.1.2.7. Master mix for PCR used in tissue specific gene expression studies

The composition of master mixture was as follows:

Contents	**Amount per 10 µl**
10X *Taq* buffer	1.0 µl
dNTP mix (10 mM)	0.5 µl
$MgCl_2$ (50 mM)	0.5 µl
Taq polymerase (1 U/ µl) (Biotool,USA)	0.5 µl
Actin forward primer (20 pM)	0.5 µl
Actin reverse primer (20 pM)	0.5 µl
cDNA (100 ng/ µl)	1.0 µl
MilliQ water	5.5 µl

3.1.2.8. Hoagland medium for growing wheat plants in hydroponics setup

The composition of Hoagland medium was as follows:

Contents	**Concentration (g/10L)**
KH_2PO_4	136
KNO_3	101
$Ca(NO_3)_2.4H_2O$	236
$MgSO_4.7H_2O$	246
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.08
$CuSO_4.5H_2O$	0.22
H_2MoO_4	0.02
Na_2EDTA	7.45
$FeSO_4.7H_2O$	5.57

3.1.2.9. Luria Broth (LB) medium

The composition for LB medium was as follows:

Contents	Amount per L
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Distilled water	1 L

3.1.2.10. Preparation of SOC medium

SOC medium was prepared by mixing 20 mM glucose, 2.5 mM $MgCl_2$ and 10 mM $MgSO_4$ in the LB medium.

3.1.2.11. Calcium chloride buffer for competent cells preparation

The composition of calcium chloride buffer was as follows:

Contents	Amount per L
Piperazine-N, N′-bis(2-ethanesulfonic acid) (PIPES)	3.021 g
$CaCl_2.2H_2O$	2.205 g
KCl	18.637 g
$MnCl_2.4H_2O$	10.885 g

3.2. Methods

3.2.1. Screening of wheat cultivars for nitrate tolerance

Seeds of eight wheat cultivars were tested for root architecture changes in presence of nitrate concentrations of 0 mM (0 %), 0.5 mM (10 %), 2.5 mM (50 %) and 5 mM (100 % or control). Ten seeds of each cultivar were sterilized with 0.1 % (w/v) $HgCl_2$ (Himedia) solution for 30 sec and thoroughly rinsed with de-ionized water. The safety measures for handling and disposal of treated water containing 0.1% $HgCl_2$ were done as per the laboratory safety manual.

The seeds were then allowed to germinate in petri dishes. The petri dishes containing the seeds were placed in a dark room at 20 ^{o}C for 5-7 days. The seedlings were transferred into 7 L plastic trays containing Hoagland medium. Plants were grown at light period of 16 h and dark period of 8 h. The day and night temperature for wheat plants were maintained at 22 ^{o}C and 18 ^{o}C, respectively. The medium was continuously aerated and changed after every 3-4 days. The data were recorded for root length, shoot length, biomass and number of lateral roots after 21 days. Each screening experiment was repeated thrice. A nitrate tolerant cultivar (WH 1021) and a nitrate sensitive cultivar (PBW 343) were selected for further experiments.

3.2.2. Determination of nitrogen (N) and carbon (C) contents

Nitrogen content in the plant samples were determined by following the protocol described by Dhaliwal and coworkers [55]. The roots of each variety from the 0 % nitrate treatment and control were freeze dried. The dried samples were sliced into very fine pieces with a sterilized blade. The weight of each sample taken for measurement was 2 mg. These samples were sealed properly with aluminium covers. The C, N and H content was determined with the help of CHNS analyser (Elementar, Germany).

3.2.3. Determination of activities of antioxidant enzymes

3.2.3.1. Preparation of plant extract

A fresh root sample (0.5 g) from wheat seedling was ground with a mortar and pestle in 2ml of extraction buffer and kept at 4 ^{o}C for 2 h. The extraction buffer was composed of 2 g polyvinylpyrrolidone (PVP), 2 ml 100 mM dithiothritol (DTT) and 400 µl 0.5 M EDTA dissolved in 100 ml of 100 mM phosphate buffer (pH 7.0). The contents were centrifuged at 12,000 x g for 25 min at 4 ^{o}C. The supernatant was used as enzyme extract in the assay of antioxidant enzymes.

3.2.3.2. Determination of superoxide dismutase (SOD) activity

The SOD activity was determined by the protocol described by Marklund and Marklund [148]. The reaction mixture consisted of 2.750 ml of 100 mM (pH 7.0) potassium phosphate buffer, 150 µl of 20 mM pyrogallol (0.025 g of pyrogallol dissolved in 10 ml of 0.1 N HCl) and

100 µl of enzyme extract. The enzyme extract was not added to the reaction mixture in the control reaction. The purpurogallin production was recorded at an absorbance of 420 nm using a UV-visible Varian spectrophotometer, model Cary 100. One unit of enzyme activity was defined as the amount of enzyme required to inhibit 50 % of pyrogallol autooxidation. The calculation of enzyme activity and specific enzyme activity was done using the following formula:

Enzyme activity (U/ml) = $(\Delta A_{420}/min) \times V_t \times d.f)/ € \times V_s$

Where V_t = total volume of reaction mixture (3 ml), d.f = dilution factor, V_s = sample volume (0.1 ml), €= Molar Extinction co-efficient (12 for purpurogallin)

Specific activity = Enzyme activity (U/ ml)/ mg protein. The concentration of protein was calculated using Bradford assay method [25].

3.2.3.3. Determination of peroxidase (POD) activity

The peroxidase activity was estimated by the method described by Park [178]. The reaction mixture (3 ml) consisted of 2.440 ml of phosphate buffer, 160 µl of H_2O_2 (one ml of H_2O_2 dissolved in 74 ml of MilliQ water), 300 µl of 5.33 % pyrogallol (5.33 g of pyrogallol dissolved in 100 ml of 0.1 N HCl) and 100 µl of enzyme extract. The purpurogallin production was recorded at an absorbance of 420 nm at 0 min and 5 min using UV-visible Varian spectrophotometer, model Cary 100. One unit of enzyme was defined as the amount of enzyme required to catalyze the production of 1 mg purpurogallin per min. The calculation of enzyme activity was done the same way as that of superoxide dismutase activity.

3.2.3.4. Determination of antioxidant activity by DPPH

The antioxidant enzyme activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay described by Brand-Williams and coworkers [26]. An aliquot of 25 µl of enzyme extract was added to 0.5 ml of 50 µM methanolic solution of DPPH in an Eppendorf tube. The reaction mixture containing enzyme extract along with appropriate control was incubated for 30 min in dark chamber. 100 µl aliquots of all the incubated samples were added to 96-well plate. The absorbance was recorded at 517 nm using a microplate reader (Biotek,

Cytation 3). The calculation of the percentage free radical scavenging activity in samples was done by using the following formula:

% Scavenging activity = (AC_{517} -AS_{517}/AC_{517} x 100)

AC and AS are the absorbance of the DPPH solution (without enzyme extract) and sample solution (with enzyme extract), respectively.

3.2.4. Primer designing for amplification of genes involved in root architecture

The genes coding for root architecture in wheat, involved directly or indirectly, were selected from the NCBI (http://www.ncbi.nlm.nih.gov), Cerealsdb (www.cerealsdb.uk.net) and Phytozome (http://www.phytozome.net) databases. The cDNA sequences for the genes were obtained from the EST database available at NCBI. With the help of these sequences, primers for amplification of root architecture genes of wheat were designed using the MBCF oligo calculator and Primer3 tool.

3.2.5. DNA extraction and purification

Roots were collected from the field grown wheat plants of Chinese spring variety and DNA was extracted by the slightly modified CTAB method described by Doyle and Doyle (1990). The 0.5 g roots were ground in liquid nitrogen to make a fine powder using pre-chilled, sterile mortar and pestle. The powder was added to a 2 ml Eppendorf tube containing 1 ml of pre-warmed DNA extraction buffer. The contents were gently mixed and incubated in a water bath at 65 ^{o}C for 1 h followed by incubation at room temperature for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the Eppendorf tube. The contents were mixed gently and centrifuged at 8000 x g for 15 min at room temperature. The upper aqueous layer was transferred carefully in to another Eppendorf tube. The DNA was precipitated by adding an equal volume of ice cold isopropanol and incubating at 4 ^{o}C for 2 h. The precipitated DNA was pelleted by centrifugation centrifuged at 8000 x g for 15 min. The pellet was washed twice with 70 % ethanol, air dried and dissolved in 70 µl TE buffer. RNA was removed by treating with 2 µl of RNaseA (10 mg/ml) and incubating at 37 ^{o}C in a water bath for 1 h. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the contents were centrifuged at 8000 x g for 15 min at room temperature. The pellet was discarded and the supernatant was

transferred to another Eppendorf tube. The DNA was precipitated by adding an equal amount of 100 % ethanol and incubating at 4 ^{o}C for 2 h. The contents were centrifuged at 8000 x g for 15 min. The supernatant was discarded and the pellet was air dried. Finally the pellet was dissolved in 70 µl TE buffer.

3.2.6. Quantification and dilution of DNA

The quality of extracted DNA was determined by gel electrophoresis on 1 % agarose gel. The isolated wheat DNA was quantified by measuring the absorbance at 260 nm in a UV-visible Varian spectrophotometer, model Cary 100 and diluted with $T_{10}E_1$ buffer to 70-100 ng/ µl. The diluted DNA samples were stored at -20 ^{o}C until use.

3.2.7. Polymerase chain reaction

The genes involved in root architecture in wheat were amplified from the total DNA of wheat using Mastercycler gradient programmable thermal cycler (Applied Biosystems®). The annealing temperature (Ta) for genes involved in the present study were as follows: *TaEXP* (57 ^{o}C), *TaGLU3* (60 ^{o}C), *TaMNSOD* (60 ^{o}C), *TaNRT2* (60 ^{o}C), *TaIAA* (57 ^{o}C), *TaActin* (57^{o}C), *TaRTCS* (60 ^{o}C), *TaPDS* (57 ^{o}C) and *BSMV* (59 ^{o}C) The PCR program was run as shown in Fig. 3.1.

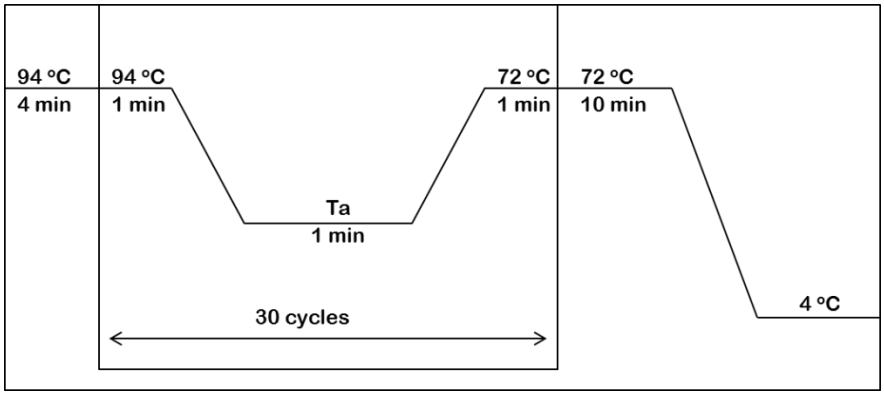

Fig. 3.1. PCR program used for DNA amplification.

3.2.8. Total RNA Isolation

For isolating total RNA, all the plasticwares/ glasswares to be used were treated with 0.1 % diethylpyrocarbonate (DEPC, Biomatik) water. The glasswares were dipped with 0.1 % DEPC water, kept overnight at 37 °C and then autoclaved. Total RNA was isolated from germinating roots and coleoptile on 7th day after sowing (DAS), seedling crown on 15th DAS, shoot on 21st DAS, flag leaf on 37th DAS and roots on 21 days post treatments with TRIzol® reagent (Ambion) using manufacturer's protocol. The fresh tissue was ground into pre-chilled mortar and pestle with liquid nitrogen and was immediately transferred to a TRIzol® (500 µl) containing fresh Eppendorf tube. The contents were vortexed and centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant (400 µl) was transferred to an Eppendorf tube and kept at room temperature for 10 min. An amount of 200 µl of chloroform was added to the supernatant. The mixture was vortexed and kept at room temperature for 5 min. The Eppendorf tube was spinned at 12000 x g for 15 min at 4 °C .The supernatant was removed and equal volume of isopropanol (500 µl) was added. The mixture was thoroughly mixed by inverting, kept at room temperature for 5 min and centrifuged at 12000 x g for 15 min at 4 °C. The supernatant was discarded and the pellet was dissolved in 1 ml of 70 % ethanol by vortexing followed by centrifugation at 7500 x g for 5 min at 4 °C. The supernatant was discarded and the pellet was air dried for 15 min. The pellet was resuspended in 30-50 µl of DEPC water and further stored at -80 °C. The isolated RNA obtained from the tissues was checked on 1 % agarose gel (SeaKem LE agarose-Lonza), visualised under UV and documented using gel documentation system. The RNA samples were quantified using nanodrop (Denovix Ds 11 spectrophotometer) and optical density at 260 nm and 280 nm (OD260/280) were recorded.

3.2.9. DNase treatment and precipitation of total RNA isolated from wheat tissues

For removing the traces of DNA in the isolated RNA, DNase I (Ambion) treatment was given to the isolated RNA using the manufacturer's instruction. 1 µg RNA (in 0.1 % DEPC water) sample was used for the treatment with the following concentrations and volume of reaction mixture

RNA (1 µg) – x µl

DNase I (2U)- 1 µl

5X DNase Buffer- 5 µl

DEPC H_2O- to make volume upto 25 µl

The reaction mixture was incubated at 37 °C for 30 min and tube was transferred immediately to ice. The purification of total RNA was done using the phenol: chloroform method. The sample treated with DNase I was mixed with EGTA (20 mM) which was 1/10th volume of total reaction. The mixture was placed in a water bath at 65 ^{o}C. After 10 min equal volume of phenol: chloroform was added in the tube. The contents were gently mixed and centrifuged at 12000 x g for 10 min. The supernatant was transferred to a new Eppendorf tube. An aliquot of sodium acetate (3 M, pH 5.2) which was 1/10th volume of total reaction and 2.5 volume of absolute alcohol was added. The reaction mixture was spinned at 12000 x g for 10 min at room temperature. The supernatant was discarded and the pellet was air dried for 15 min. The pellet was resuspended in 30-50 µl of DEPC water and further stored at -80 °C.

3.2.10. Single stranded complementary DNA (cDNA) synthesis

An aliquot of 1 µl RNA sample (1 µg), 1 µl Oligo dT (Sigma, 2.5 mM) primer and 11 µl of 0.1 % DEPC treated MilliQ H_2O were mixed in a PCR tube. The PCR tube containing the reaction mixture was placed in a water bath at 65 ^{o}C. After 10 min the tube was taken out and kept at room temperature for 5 min. The reaction mixture was kept in a thermo cycler (Applied Biosystems®) and single stranded complementary DNA was synthesized using one RT-PCR cycle (10 min at 25 ^{o}C followed by 60 min at 42 ^{o}C and finally at 9 ^{o}C for 2 min) for the 20 µl reaction mixture. The synthesized cDNA was stored in -20 ^{o}C for further analysis.

3.2.11. Semi-quantitative PCR amplification of cDNA

The Semi-quantitative PCR was performed in the wheat tissues as described by Abouseadaa and coworkers [1]. The 10 µl PCR reaction mixture contained 1 µl synthesized cDNA samples, a primer pair set for the gene and the master mix. After denaturation at 94 ^{o}C for 5 min, each sample was subjected to 30 cycles (1 cycle: 1 min at 94 ^{o}C, 1 min at respective Ta, 1 min at 72 ^{o}C) of amplification and a final extension of 10 min at 72 ^{o}C. *TaActin* was used as an internal control. The amplified PCR product was visualized on 1.2 % agarose gel and quantification was done by calculating the intensity of band using IMAGE 4 software as described by Leblanc *et al*. [130] and Bhushan *et al.* [21] .

3.2.12. Retrieval of *RTCS* gene sequence

For the comparative and evolutionary studies of *RTCS* gene among plants, full length gene/ cDNA sequences of *RTCS* gene for *Sorghum bicolor*, *Setaria italica*, *Panicum virgatum*, *Oryza sativa*, *Arabidopsis thaliana*, *Solanum tuberosum*, *Solanum lycopersicum*, *Glycine max*, *Capsella rubella and Brassica rapa* were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) and Phytozome (http://www.phytozome.net/) databases. Since genomic sequence of wheat is not known, orthologs for *RTCS* gene of wheat was retrieved from NCBI EST database and full length putative gene sequence was predicted using 5X Chinese spring wheat contigs data available at Cerealsdb (www.cerealsdb.uk.net) database. The EST of putative *RTCS* gene of wheat extracted from NCBI was used as a query in 5X genome sequence choosing assembled and unassembled reads. The reads obtained were assembled using CAP3 option tool and the complete sequence was obtained using the overlaps of bases in the unassembled reads. The protein sequence was derived from the consensus gene sequence obtained by EXPASY translate (http://web.expasy.org/translate) tool.

3.2.13. Prediction of *RTCS* gene structure

The exons and introns in various *RTCS* gene sequences were identified by comparing cDNA sequences with genomic DNA sequences by using BLAST tool of NCBI and Fgenesh program. The exact locations of exons and introns were found out by comparison of the results obtained from the two programs. The exons of *RTCS* gene of each species were aligned using vector NTI software and similarity of each sequence with the *RTCS* gene of maize was determined.

3.2.14. Analysis of amino acid sequences of *RTCS* gene orthologs

The predicted amino acid sequences of all the species were aligned using CLC main-workbench (http://www.clcbio.com/) alignment tool. The sequence of six monocots and six dicots were compared with the resulting consensus sequence to determine the sequence similarity at each position of protein. A score of 6 for monocots and dicots represented the complete conservation and score of 0 represented no similarity in comparison to the consensus sequence. The conserved domain database (CDD) tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/

wrpsb.cgi) search was used to identify the domains in the consensus sequence of RTCS protein. The motifs in the domain obtained were identified by PredictProtein tool [193].

3.2.15. 3-D structure prediction and ligand binding

The 3Dpro of SCRATCH server (http://www.igp.uci.edu) [36] and iTASSER tool (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) [194] was used to predict the three dimensional (3D) structure of RTCS protein because RTCS protein sequence have not good structural templates for homology modeling. Here tertiary structure prediction methods are evaluated by the threader-based approach using Critical Assessment of Structure Prediction (CASP). The predicted models were evaluated using SAVES server (https://services.mbi.ucla.edu/SAVES/) and PROCHECK tool and the model having minimum number of residues in the disallowed region was selected. ProSA-web tool (https://prosa.services.came.sbg.ac.at/prosa.php) was used to calculate the Z score of the predicted model [235]. The protein model for TaRTCS generated by SCRATCH server was used to predict ligand-binding side by COACH server (http://zhanglab.ccmb.med.umich.edu/COACH/) [245]. The interaction between protein and ligands was visualized by PyMOL software [51].

3.2.16. Phylogenetic analysis

All the amino acid sequences of RTCS protein retrieved from different species were loaded on ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) tool to determine the phylogeny. NJdist and ProtML programs in the MOLPHY package [2] were used to determine the Maximum Likelihood tree. The evolutionary relationship was inferred by neighbour joining algorithm of distant matrix. The local bootstrap probability was estimated by using ProtML program. A phylogenetic tree along with boot strap values was constructed using MEGA 6.0 software (http://ab.inf.uni-tuebingen.de/software/megan/).

3.2.17. Molecular evolution analysis

Molecular evolution analysis of *RTCS* gene in all the species was conducted by using DnaSP ver. 5.0 tool [134]. The number of segregating polymorphic sites θ [231] and the average number of nucleotide differences per site between two sequences π [168] were calculated to

estimate the nucleotide diversity. Tajima's D statistic was used for tests of neutrality. CpG islands in all monocot and dicot RTCS nucleotides were predicted by using TOUCAN3 [3] software package.

3.2.18. Physical mapping in wheat

The ESTs and CDSs of wheat *RTCS* gene were used to physically localize the gene on wheat chromosome using the mapped EST database (http://wheat.pw.usda.gov/GG3/blast) using the default parameters. To accurately map wheat *RTCS* gene, ESTs representing this gene were aligned against the survey sequence data and only those hits were considered which showed more than 90 % sequence similarity and 80 % query coverage. Further, deletion lines for all the chromosomes of wheat were used for the verification of *in-silico* mapping.

3.2.19. Virus induced gene silencing of *RTCS*

3.2.19.1. Preparation of empty vector

Three plasmids (pα, pβΔβa, and pγ) derived from BSMV were used in this study as described by [198]. For preparing the empty vector, bPDS4 cloned gamma plasmid provided by Dr. Kulvinder Singh Gill (Prof. at WSU, USA) was used (Fig. 3.2.19.1). As bPDS4 insert consist of *Not*I and *Pac*I restriction sites so the restriction digestion of gamma vector was done by the following reaction:

Plasmid DNA- 10 µl (15 µg)
Buffer 2 (10X) - 2.0 µl
Not I (20 U/ µl) - 2.0 µl
Pac I (10 U/ µl) - 2.0 µl
BSA (100X) - 0.2 µl
Water - 3.8 µl
Total reaction volume - 20.0 µl

The reaction mixture was incubated at 37 ^{o}C for 4 h. All the restriction enzymes and BSA were purchased from New England Biolabs. The double digested product was loaded on 0.8 % agarose gel along with molecular weight marker. The desired fragment (empty vector) was cut from the gel under UV light with the help of surgical blade and transferred into 2.0 ml Eppendorf tube. The weight of the gel piece was measured by calculating the initial and final weight of the Eppendorf tube. Gel purification kit (Invitrogen) was used to get the purified vector DNA as per the manufacture's instruction. The concentration of linearized plasmid was estimated using the λ DNA (10 ng/ µl) concentration marker on 0.8 % agarose gel.

3.2.19.2. Barley stripe mosaic virus (BSMV) based VIGS constructs

Infectious RNAs obtained from three BSMV-based plasmids were used in all the gene silencing experiments. Two plasmids, pα and pβΔβa, were common in all the experiments. The gene silencing construct of *RTCS* gene was inserted in the third plasmid pγ and the resulting plasmid obtained was named as pγ.*TaRTCSas*. A 100 bp unique region of gene was selected for designing of gene silencing construct (pγ.*TaRTCSas*) as reported by previous workers [15]. Two complementary oligonucleotides corresponding to above selected region with added restriction sites (*Pac*I on 5' and *Not*I on the 3' ends) were synthesised commercially (Sigma Aldrich, USA). The sequences of these nucleotides in antisense orientation were as follows:

RTCS-AS F

5'TAAGCGCTGTGGCGCCGCTGTAGCAGCCGGACTGCTCTGCGTGGACGTCATGGACT
GGTGGTCGTGCTGATCCGCGGCGGCGGCCACCTGC 3'

RTCS-AS R

5'GGCCGCAGGTGGCCGCCGCCGCGGATCAGCACGACCACCAGTCCATGACGTCCAC
GCAGAGCAGTCCGGCTGCTACAGCGGCGCCACAGCGCTTAAT 3'

The two complementary synthetic oligonucleotides of RTCS were suspended in 0.1X TE buffer at a final equimolar concentration of 10 µg/µl followed by heating at 65 °C for 20 min and incubation at room temperature for 1 h to obtain annealed oligonucleotides. Annealed construct

was ligated in the γ cloning vector digested with *Pac*I and *Not*I restriction enzymes. The composition of annealing mixture is given below:

Content	**Amount (5 µl)**
Vector (25 ng/µl)	1.0 µl
Insert (25 ng/µl)	3.0 µl
Buffer (10X)	0.5 µl
T4 Ligase (400 U/µl)	0.5 µl

The reaction mixture was placed in a PCR machine with programme set for 16 °C for 18 h. Fig. 3.2. shows the vector map along with PDS insert in gamma plasmid. The plasmid construct was purified by agarose gel electrophoresis.

The gene silencing construct bPDS4 was inserted in plasmid pγ and the resulting plasmid obtained (pγ.bPDS4) was used as positive control. A 121 bp fragment derived from multiple cloning site (MCS) of pBluescript K/S (Stratagene) vector was inserted in pγ plasmid and the resulting plasmid γMCS, was used as a negative control containing "virus only" [96].

3.2.19.3. Preparation of ultra-pure competent cells

The ultra-pure competent cells were prepared by following the protocol described by Okamoto *et al*., [173]. LB plates were made by adding 15 g/L bacto agar into the LB medium. The pH of the medium was adjusted to 7.0 using 0.1 N NaOH. The medium was autoclaved at 15 *psi* pressure and 121 °C temperature. The mixture was allowed to cool to 55 °C after autoclaving and 100 µg/ml ampicillin was added. The bacterial cells were revived from glycerol stock by streaking on LB plates. The plates were incubated overnight at 37 ^{o}C. Single colony was transferred into 25 ml of SOC medium and kept at 200 rpm incubator- shaker (Kuhner LT-X, Lab-Therm) for 6-8 h at 37 ^{o}C until an OD_{600} of 0.2-0.4 was obtained. One ml of primary culture was added into fresh 25 ml of SOC medium which was kept in incubator shaker at 200 rpm for 3-4 h at 37 ^{o}C. The cells were harvested by centrifugation at 4000 rpm for 10 min at 4 ^{o}C, then resuspended in 20 ml of sterile cold $CaCl_2$ solution and incubated on ice for 30 min. The mixture was again centrifuged as above and the resulting cell pellet was resuspended in 4 ml of sterile

cold $CaCl_2$ solution to yield the final competent cell suspension. These competent cells were used for transformation.

3.2.19.4. Transformation of annealed product

One µl of ligated mixture was used to transform chemically competent *E. coli* cells by giving a heat shock at 42 °C. The cells were spread on LB agar medium plate containing 100 µg/ml ampicillin and incubated overnight at 37 °C in an incubator. Single transformed colony was picked and cultured in 2 ml LB medium. The culture was kept overnight at 37 ^{o}C in an incubator shaker. Plasmid isolation was done using PureLink Quick plasmid mini prep kit (Invitrogen, USA) as per the manufacturer's instruction. Validation of colonies was done by using colony PCR and restriction digestion of plasmid with *Not* I and *Pac* I enzyme. Sequencing of the plasmid was done from commercial sources (Thermo Fischer, Gurgaon) using ABI sequencer for validating the true insert.

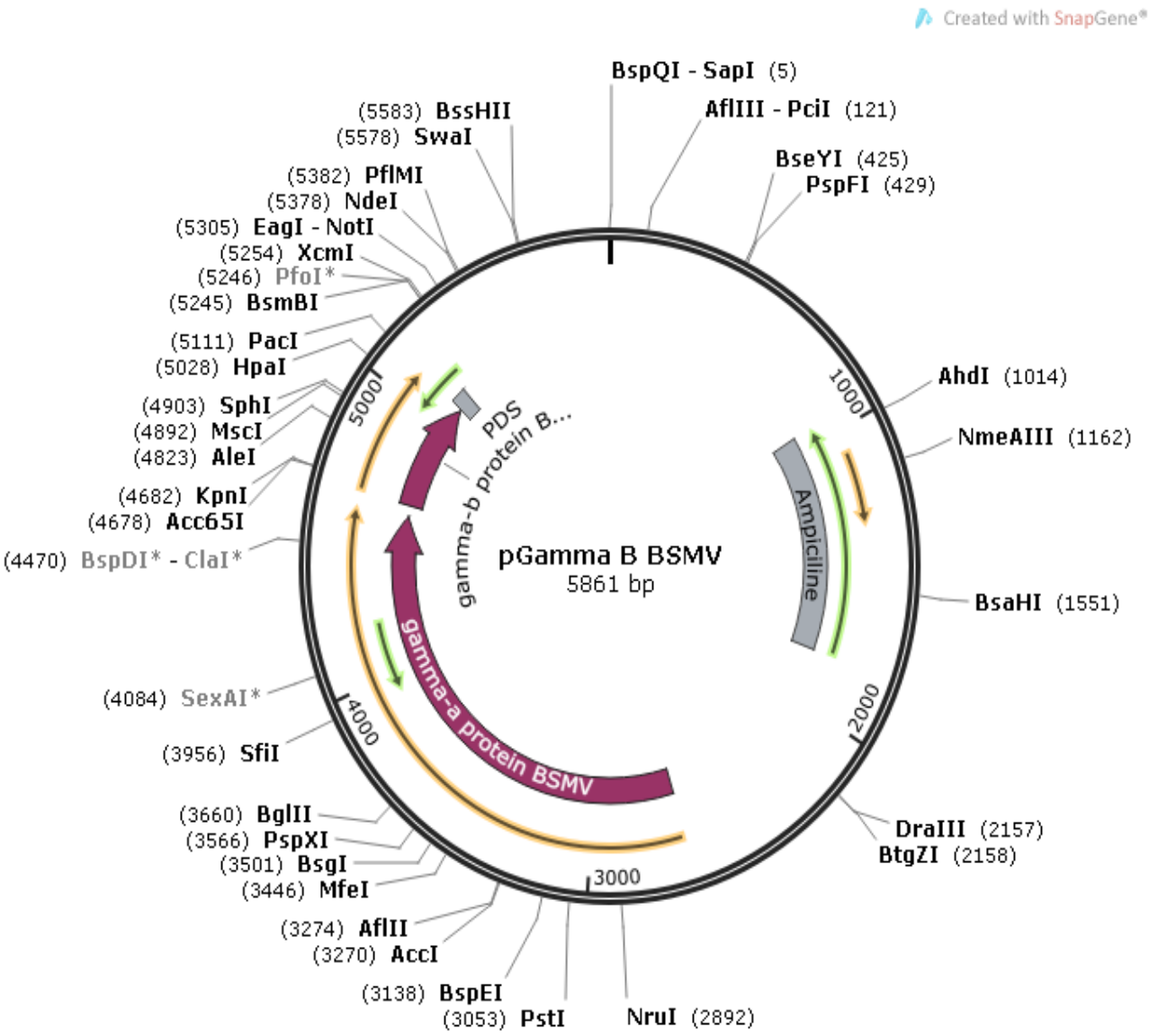

Fig. 3.2. Vector map of bPDS4- gamma BSMV construct

3.2.19.5. *In vitro* transcription

Plasmids, pα, pβΔβa, and pγ.MCSas/ pγ.bPDS4as/ pγ.TaRTCSas were digested with *Mlu*I, *Spe*I and *Bss*HII, respectively as mentioned in Table 3.1. Infectious RNAs were produced by *in vitro* transcription of digested plasmids using Ambion®mMESSAGEmMACHINE® kit (Life Technologies Corporation, NY, USA) following manufacturer's instructions.

Table. 3.1. Composition of reaction mixture used for linearization of three plasmids

SNo.	pα	pβΔβa	pγ	Amount (µl)
1	α plasmid	β plasmid	γ plasmid	20
2	Buffer3	Buffer 2	Buffer 3	3
3	*Mlu* I(10U/µl)	*Spe* I (10U/µl)	*BssH*II (5U/µl)	2
4	dH_2O	dH_2O	dH_2O	5
			Total reaction Volume	30

The reaction mixture containing α and β plasmids were incubated overnight at 37 ^{o}C whereas all the γ plasmids (PDS, RTCS and MCS) were kept overnight at 50 ^{o}C in an incubator.

3.2.19.6. Plant growth conditions

Wheat plants of cultivar Chinese spring were propagated in 4x4 inches pots using Turface MVP mixture (PROFILE Products LLC, USA). Plants were grown in growth chamber under 16 h light and 8 h dark period with a constant day (24 °C) and night (18 °C) temperature. Plants were watered with Hoagland nutrient solution every alternative day.

3.2.19.7. Plant inoculations

The three infectious RNA preparations (pα, pβΔβa and pγ) were mixed in 1:1:1 ratio. This RNA preparation (10 µl) was used to inoculate 2nd leaf of a ten days old wheat plant by following the procedure as described by [15]. For study of *RTCS* gene silencing in roots, ten seedlings were inoculated with infectious virus RNAs carrying gene of interest (*RTCS*). Besides gene of interest, inoculations were also done with infectious virus carrying MCS (5 seedlings) and bPDS4 (5 seedlings) and FES (5 seedlings; as infection control). Five seedlings were kept uninoculated. The experiment was repeated three times.

3.2.19.8. Phenotyping of silenced plants

The viral disease symptoms in the form of mosaic patches were recorded for each infected plant after 21 days of inoculation. The characters such as root length, root density, shoot length, number of tillers and color of leaves were also recorded for individual plant.

3.2.19.9. Sample collection

A leaf sample was obtained by cutting the fourth leaf of an experimental plant at 21 days post inoculation stage. For collecting the root sample an experimental plant was gently removed from Turface. The root of the plant was cut with a razor after washing with autoclaved water. Each plant sample was immediately frozen in liquid nitrogen and stored at -80 °C.

3.2.19.10. Quantitative real time-PCR

The quantitative gene expression was studied by using the High resolution melting master mix kit (Roche) as per the manufacturer's instructions with the modification of changing the concentration of $MgCl_2$ to 4 mM in the cocktail mixture. The housekeeping gene, β-actin was used as a reference gene in calculating the relative quantification of the gene of interest. The PCR conditions were as follows: 95 °C for 5 min; followed by 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 40 sec and a final step at 72 °C for 5 min. Three technical replicates and 10 biological replicates were performed for each PCR primer. Normalisation of the amount of gene specific transcripts accumulated/silenced was carried out using accumulation values (intensities) of *TaActin* as internal controls for each root and leaf tissue [141]. Presence of BSMV in all samples was monitored by using the γ-Flanking MCS primers (annealing temperature 62 °C)(Table 4.9). VIGS was expressed as the ratio of TaRTCS transcript accumulation (normalized to β-actin transcript accumulation) in γ.RTCS-inoculated seedlings to that in γ.MCS-inoculated seedlings. Similarly, gene silencing/knock down of PDS gene was calculated as a ratio of bPDS4 transcripts accumulation (normalized to TaActin transcript accumulation) in γ.bPDS4a-inoculated seedlings to that in γ.MCS and FES-inoculated plants.

3.2.20. IAA treatment and expression analysis

The roots of Chinese spring seedlings (10 day-old) were treated with 1μM indole 3-acetic acid (IAA) and samples of plants were taken after 1 h, 3 h, 6 h, 12 h and 24 h. Semi-quantitative RT-PCR was performed to study the expression of *TaIAA* and *TaRTCS* genes using the gene specific primers.

3.2.21. Statistical analysis

The data was analysed by one-way ANOVA (analysis of variance) and the treatment mean values were compared by Tukey's Post HOC test ($p \leq 0.05$) using Statistical Package for the Social Sciences [SPSS] software; version17, while two-way ANOVA was done with Tukey's multiple comparisons test between groups using GraphPad Prism 6.0 software.

Chapter 4

Results

4. RESULTS

4.1. Screening of wheat cultivars for nitrate stress tolerance

The data showing the root lengths of eight wheat cultivars grown under different nitrate concentrations have been presented in Table 4.1. Two wheat cultivars, PBW343 and WH542, showed significant differences in the root lengths as compared to the control (5 mM) at nitrate concentrations of 0 mM and 0.5 mM. The root length of PBW343 cultivar at nitrate concentration of 0 mM was 13.875 cm, which was significantly higher than that of the control plants (7.8 cm). No significant difference in the root length of wheat cultivar WH1021 was observed as compared to the control at 0 mM nitrate concentration.

The shoot lengths in eight wheat cultivars under different nitrate concentrations have been presented in Table 4.2. All wheat cultivars showed no significant difference in the shoot lengths at different nitrate concentrations as compared to the control.

The data showing the root length/shoot length ratios in eight wheat cultivars under different nitrate concentrations have been presented in Table 4.3. Significant differences in the root length/shoot length ratios as compared to control (5 mM) were observed at nitrate concentrations of 0 mM and 0.5 mM in two wheat cultivars, PBW343 and DBW17. However, no significant difference in the root length/shoot length ratio was observed at nitrate concentrations of 0 mM, 0.5 mM and 2.5 mM as compared to the control in the wheat cultivars, WH1021 and WH1025.

The data on the numbers of lateral roots in eight wheat cultivars under different nitrate concentrations have been presented in Table 4.4. It was observed that the number of lateral roots of DBW17 cultivar was 43 at 0 mM nitrate concentration whereas significantly less number of lateral roots (29) was found in the control (5 mM) plants. A significant difference in number of lateral roots at a nitrate concentration of 0 mM was also observed in PBW343 wheat cultivar as compared to the control. The wheat cultivar WH1021 did not significantly differed in the number of lateral roots as compared to control at 0 mM nitrate concentration.

Based on the above-mentioned results, one nitrate stress sensitive cultivar PBW343 and one nitrate stress tolerant cultivar WH1021were selected for further studies. The root architecture traits and phenotypes of these cultivars in response to different nitrate concentrations have been shown in Fig. 4.1. and Fig. 4.2., respectively.

Table. 4.1. Root lengths (in cm) of eight wheat cultivars under different concentrations of nitrate.

Cultivar	Nitrate concentration (0 mM)	Nitrate concentration (0.5 mM)	Nitrate concentration (2.5 mM)	Nitrate concentration (5 mM)
PBW343	13.875a±0.869	12a±0.881	10.625b±0.772	7.812c±0.582
WL711	17.350a±1.135	15.710a±-0.901	16.050a±1.083	13.750b±0.811
PBW621	11.350a±0.347	10.500a±0.721	10.450a±0.963	10.050a±0.219
WH542	11.100a±1.092	9.050a±0.743	8b±1.024	7.300b±0.997
PBW550	9.650a±0.512	9.550a±0.725	8.100a±0.843	7.600b±0.450
DBW17	12.120a±1.078	11.250a±0.395	8.500b±0.924	8.680b±0.842
WH1025	10.125a±0.757	9.125a±0.744	9.125a±1.074	7.250b±0.955
WH1021	13.750a±0.920	11.937a±0.951	11.562a±0.804	11.750a±0.976

Each value is the mean ± SD (in 8 replicates) and letters represent the significant difference ($p<0.05$)

Table.4.2. Shoot lengths (in cm) of eight wheat cultivars under different concentrations of nitrate.

Cultivar	Nitrate concentration (0 mM)	Nitrate concentration (0.5 mM)	Nitrate concentration (2.5 mM)	Nitrate concentration (5 mM)
PBW343	24.125a±2.696	26.375a±2.138	26.125a±1.916	27.750a±2.023
WL711	23.550a±2.443	25.150a±1.726	24.750a±2.211	24.200a±1.690
PBW621	25.950a±2.545	25.900a±1.169	27a±2.060	27.200a±2.510
WH542	30.400a±2.366	28.650a±1.422	27.100a±2.026	28.300a±1.019
PBW 550	25.600a±2.435	26.400a±2.005	27.400a±2.836	25.850a±1.607
DBW17	30a±1.810	29.750a±1.908	28.937a±1.596	30.500a±2.267
WH1025	29.312a±2.419	29a±2.237	29.562a±1.953	29.875a±1.125
WH1021	30.375a±2.326	30.437a±2.194	30.375a±1.622	29.750a±0.886

Each value is the mean ± SD (in 8 replicates) and letters represent the significant difference ($p<0.05$)

Table. 4.3. Root length/Shoot length ratios of eight wheat cultivars grown under different concentrations of nitrate.

Cultivar	Nitrate concentration (0 mM)	Nitrate concentration (0.5 mM)	Nitrate concentration (2.5 mM)	Nitrate concentration (5 mM)
PBW343	0.575a±0.023	0.454bc±0.032	0.406bcd±0.013	0.281d±0.017
WL711	0.736a±0.089	0.624b±0.016	0.648b±0.057	0.568b±0.075
PBW621	0.437a±0.027	0.405a±0.047	0.387b±0.078	0.369b±0.013
WH542	0.365a±0.025	0.315b±0.037	0.295b±0.024	0.257c±0.012
PBW 550	0.376a±0.033	0.361a±0.046	0.295b±0.041	0.294b±0.013
DBW17	0.404a±0.014	0.378a±0.036	0.293b±0.092	0.284b±0.012
WH1025	0.346a±0.051	0.314a±0.024	0.308ab±0.040	0.242b±0.019
WH1021	0.453a±0.059	0.392a±0.046	0.381a±0.062	0.395a±0.021

Each value is the mean ± SD (in 8 replicates) and letters represent the significant difference ($p<0.05$)

Table. 4.4. Numbers of lateral roots of eight wheat cultivars grown under different concentrations of nitrate.

Cultivar	Nitrate concentration (0 mM)	Nitrate concentration (0.5 mM)	Nitrate concentration (2.5 mM)	Nitrate concentration (5 mM)
PBW343	34.750a±1.872	30.752ab±3.654	27abc±3.154	20.120c±2.611
WL711	38.723a±4.667	35.300a±6.124	28.300b±3.186	26.100b±4.817
PBW621	35a±2.535	33.750a±3.548	32.375a±2.029	29.500a±1.228
WH542	38.375a±2.875	36.625a±3.172	31b±1.309	24c±4.407
PBW550	32.800a±2.413	32a±3.093	31.7a±3.198	27.8b±1.092
DBW17	43.250a±3.654	36.250b±3.127	31.875bc±1.831	29.250c±2.488
WH1025	28.525a±2.062	25.750a±1.549	24.125a±1.763	22.375b±2.426
WH1021	27.750a±2.583	26.750a±2.327	24.375a±2.669	24.125a±1.836

Each value is the mean ± SD (in 8 replicates) and letters represent the significant difference ($p<0.05$)

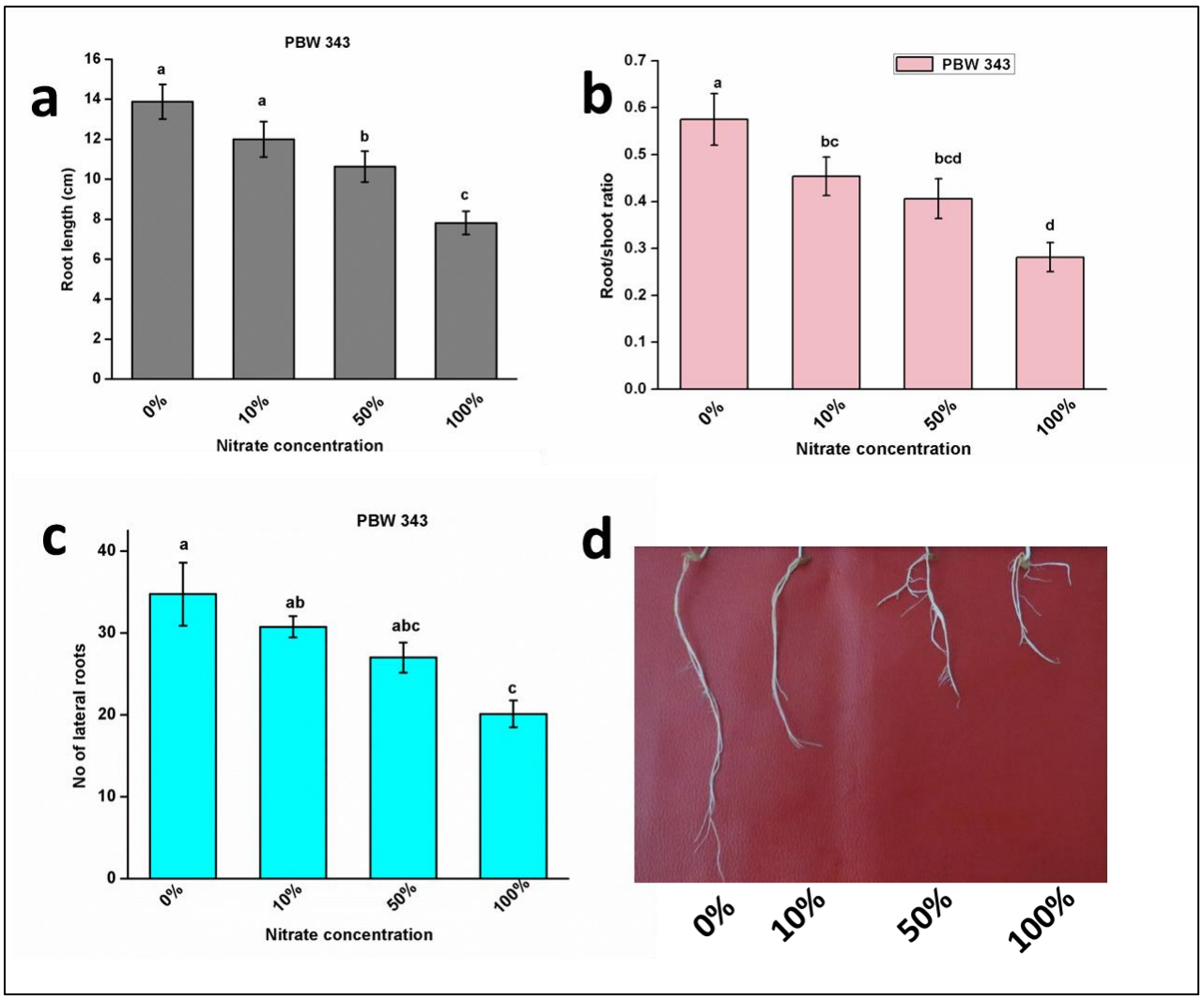

Fig. 4.1. Effect of various nitrate concentrations on the characteristics of wheat cultivar PBW343 a) root lengths, b) root length/shoot length ratios, c) numbers of lateral roots and d) phenotypes of roots. The nitrate concentration (5 mM) was used as 100 % in this experiment. Each graph value represents the mean ± SD (of 8 replicates) and letters represent the significant difference (*p<0.05*).

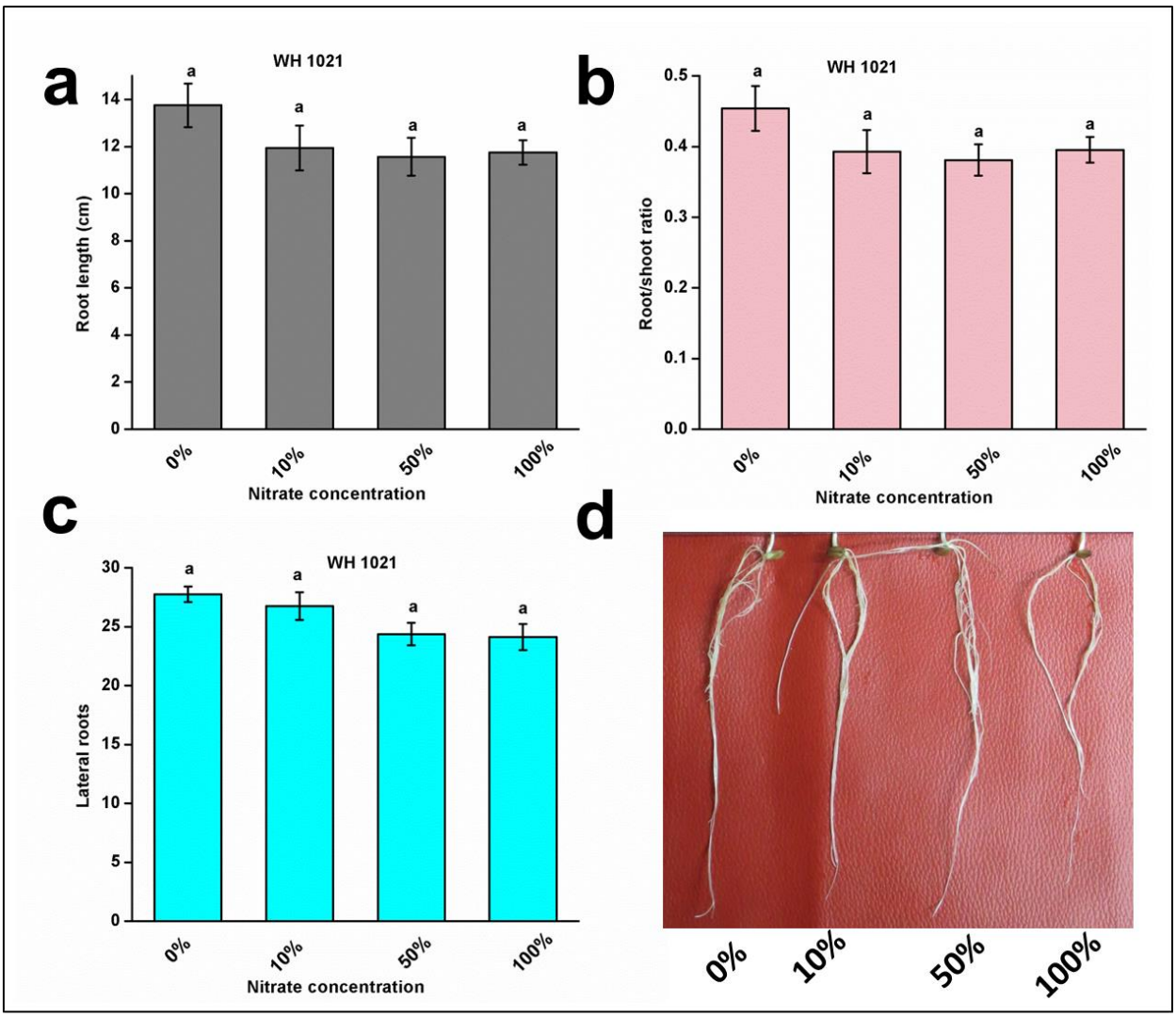

Fig. 4.2. Effect of various nitrate concentrations on the characteristics of wheat cultivar WH1021 a) root lengths, b) root length/shoot length ratios, c) numbers of lateral roots and d) phenotypes of roots. The nitrate concentration (5 mM) was used as 100 % in this experiment. Each graph value represents the mean ± SD (in 8 replicates) and letters represent the significant difference (*p<0.05*).

4.2. Nitrogen and carbon contents in roots of selected wheat cultivars

Data showing the nitrogen content, carbon content, hydrogen content and C/N ratio in the roots of two wheat cultivars, WH1021 and PBW343, at a nitrate concentration of 0 mM and control have been presented in Table 4.5. A decrease of 35 % in nitrogen content was observed in the roots of nitrate sensitive cultivar PBW343 at 0 mM nitrate concentration as compared to that of the control. However, in the roots of nitrate stress tolerant cultivar WH1021, the nitrogen content was found to decrease by 25 % at 0 mM nitrate concentration as compared to the control (Table 4.5.). The carbon content in both the cultivars was similar. The carbon to nitrogen (C/N) ratio was higher (8.5) in cultivar WH1021 as compared to cultivar PBW343 (7.3) at nitrate stress of 0 mM concentration.

Table. 4.5. Data showing the content of elements found in the roots of wheat cultivars grown at 0 mM nitrate concentration

Name of cultivar (Per 2 mg dry weight)	N (%)	C (%)	H (%)	C/N (ratio)
WH1021 (T)	4.6	39.48	5.34	8.5773
WH1021 (Control)	6.21	37.52	5.25	6.0446
PBW 343 (T)	3.19	39.87	5.45	7.3046
PBW343 (Control)	4.89	37.71	5.52	6.8292

T denotes treated sample

4.3. Activities of antioxidant enzymes in roots of selected wheat cultivars under nitrate stress

The data on the specific activities of peroxidase enzyme in the roots of two wheat cultivars, PBW343 and WH 1021, have been presented in Fig. 4.3. At 0 mM nitrate concentration a significant decrease of 23 % was observed in the specific activity of peroxidase (POD) enzyme in the roots of nitrate sensitive PBW343 cultivar as compared to that of the control (5 mM). A significant increase of 30 % in the specific activity of POD was observed at 0 mM nitrate concentration as compared to that of control in the roots of nitrate stress tolerant WH1021 cultivar.

The Fig. 4.4. shows the specific activities of superoxide dismutase enzyme in the roots of two wheat cultivars, PBW343 and WH 1021. A significant increase of 66.6 % was found in the specific activity of superoxide dismutase (SOD) enzyme in the roots of nitrate sensitive WH1021 cultivar at 0 mM nitrate concentration as compared to the control (5 mM). The specific activity of SOD enzyme in the roots of nitrate sensitive PBW343 cultivar at 0 mM nitrate concentration was significantly less as compared to control plants.

The percent scavenging activities of antioxidant enzymes in the roots of two wheat cultivars, PBW343 and WH1021, have been shown in Fig. 4.5. A significant increase of 56.8 % in scavenging activity was observed in the roots of nitrate tolerant WH1021 cultivar at 0 mM nitrate concentrations as compared to the roots of control (5 mM) plants (Fig. 4.5.). No significant difference in the scavenging activity of antioxidant enzymes was observed in the roots of nitrate sensitive PBW343 cultivar at different nitrate concentrations.

The above results indicated that there was a significant increase in the specific activity of antioxidant enzymes in the roots of nitrate tolerant WH1021 cultivar at the nitrate concentration of 0 mM as compared to the control.

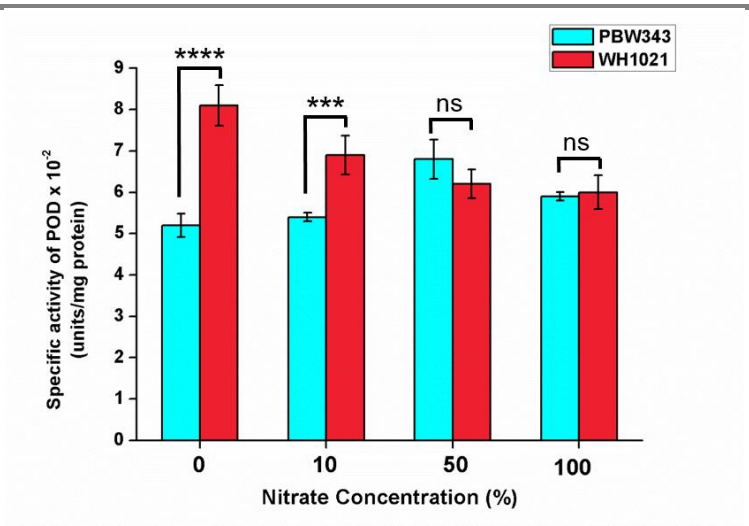

Fig. 4.3. A histogram showing the specific activities of peroxidase enzyme in the roots of PBW343 and WH1021 cultivars of wheat at various nitrate concentrations. Nitrate concentration (5 mM) was used as 100 % in this experiment. The error bars are shown as the standard deviation of the means. Statistical significant values were denoted by * (*p* < *0.001),* and **** (*p* < *0.0001*). Statistically insignificant values were represented by 'ns'.**

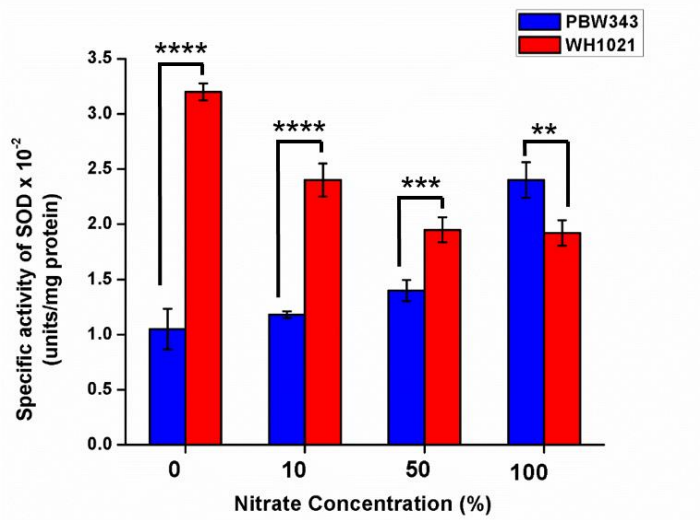

Fig. 4.4. A histogram showing the specific activities of superoxide dismutase enzyme in the roots of PBW343 and WH1021 cultivars of wheat at various nitrate concentrations. Nitrate concentration (5 mM) was used as 100 % in this experiment. The error bars are shown as the standard deviation of the means. Statistical significant values were denoted by ** (*p* < *0.005*), * (*p* < *0.001*) and **** (*p* < *0.0001*). Statistically insignificant values were represented by 'ns'.**

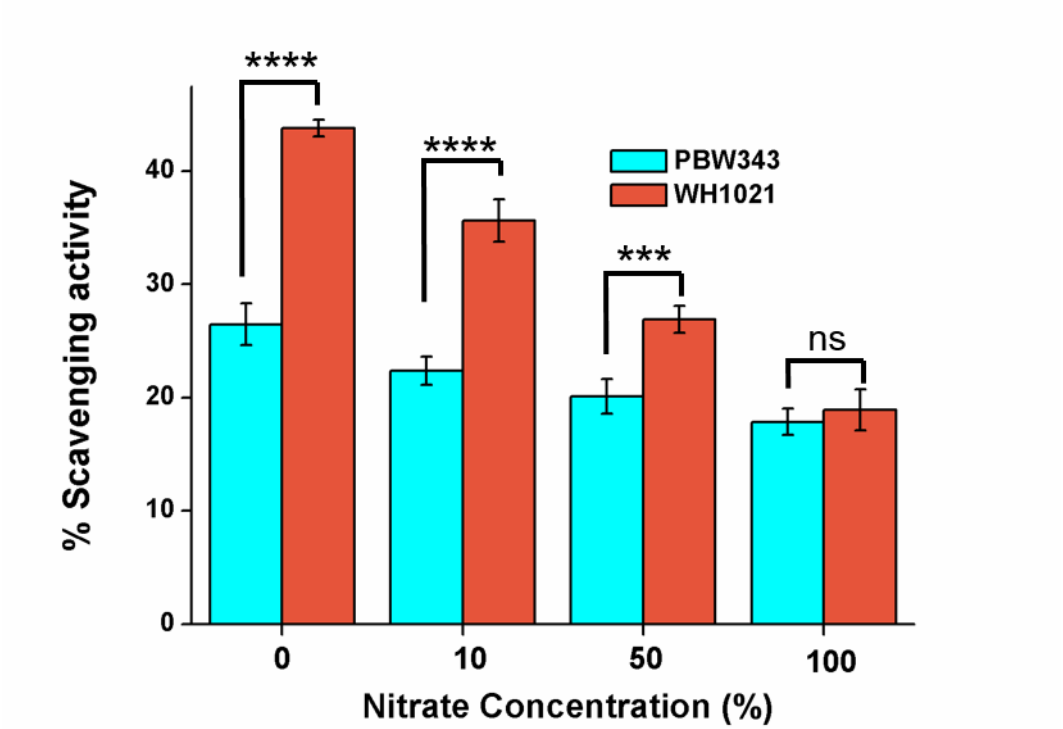

Fig. 4.5. A histogram showing the percent scavenging activities of antioxidant enzymes in the roots of PBW343 and WH1021 cultivars of wheat at various nitrate concentrations. Nitrate concentration (5 mM) was used as 100 % in this experiment. The error bars are shown as the standard deviation of the means. Statistical significant values were denoted by * (*p < 0.001),* and **** (*p < 0.0001*). Statistically insignificant values were represented by 'ns'.**

4.4. Expression profiling of root architecture related genes of wheat cultivars

Genes responsible for root architecture modification were studied in the present study on both nitrate stress sensitive (PBW343) and tolerant (WH1021) wheat cultivars. Expression profile of four genes namely *TaGLU3, TaMnSOD, TaNRT2* and *TaEXP* is shown in Fig. 4.6. Semi-quantitative PCR results of all the four genes along with internal control (*TaActin*) showed the relative expression of each gene in both wheat cultivars as bands of amplified products on agarose gel (Fig. 4.6-a). The results indicated that *TaGLU3* gene expression was more in PBW343 cultivar at 0mM nitrate concentration. However, the expression of *TaGLU3* gene appears to be constant in all the nitrate stress treatments in WH1021 cultivar (Fig. 4.6-b). Out of all the four genes, only *TaMnSOD* expression appears to be more abundant in WH1021 cultivar as compared to the PBW343 variety. However, the expression of this gene appears to be higher in both the cultivars during nitrate stress as compared to control (Fig. 4.6-c). The expression of *TaNRT2* gene appears to be highest in terms of folds out of all the genes studied. The expression was 3.5 folds more in the PBW343 cultivar at 0.5 mM nitrate concentration as compare to WH1021cultivar (Fig. 4.6-d). The expression of *TaEXP* gene was 2.5 folds more in sensitive variety at 0 mM nitrate concentration (Fig. 4.6-e). The results concluded that all the aforementioned genes showed expression differences in two wheat cultivars grown at different nitrate concentrations.

4.5. *In-silico* identification of true orthologs of *ZmRTCS* gene

Since the function of *RTCS* gene was known in maize (Taramino *et al*., 2007), the longest possible reading frame of predicted ZmRTCS protein sequence was used as a query to identify its true orthologs from other plant species, as described in material and methods section. The orthologous sequences from *Arabidopsis* and rice were available at TAIR and RAPDB databases, respectively (Shuai *et al*., 2002; Inukai *et al*., 2005). The *RTCS* orthologous sequences from other plant species were identified by tBLASTn tool with default parameters. A true ortholog was identified for each species that showed an E-value of 0 along with the highest level of coverage and identity throughout the sequence length. Since the full length *RTCS* ortholog was not

available in the wheat database, a complete orthologous genomic sequence was assembled by combining various available genomic sequences including Chinese Spring 5X genomic database.

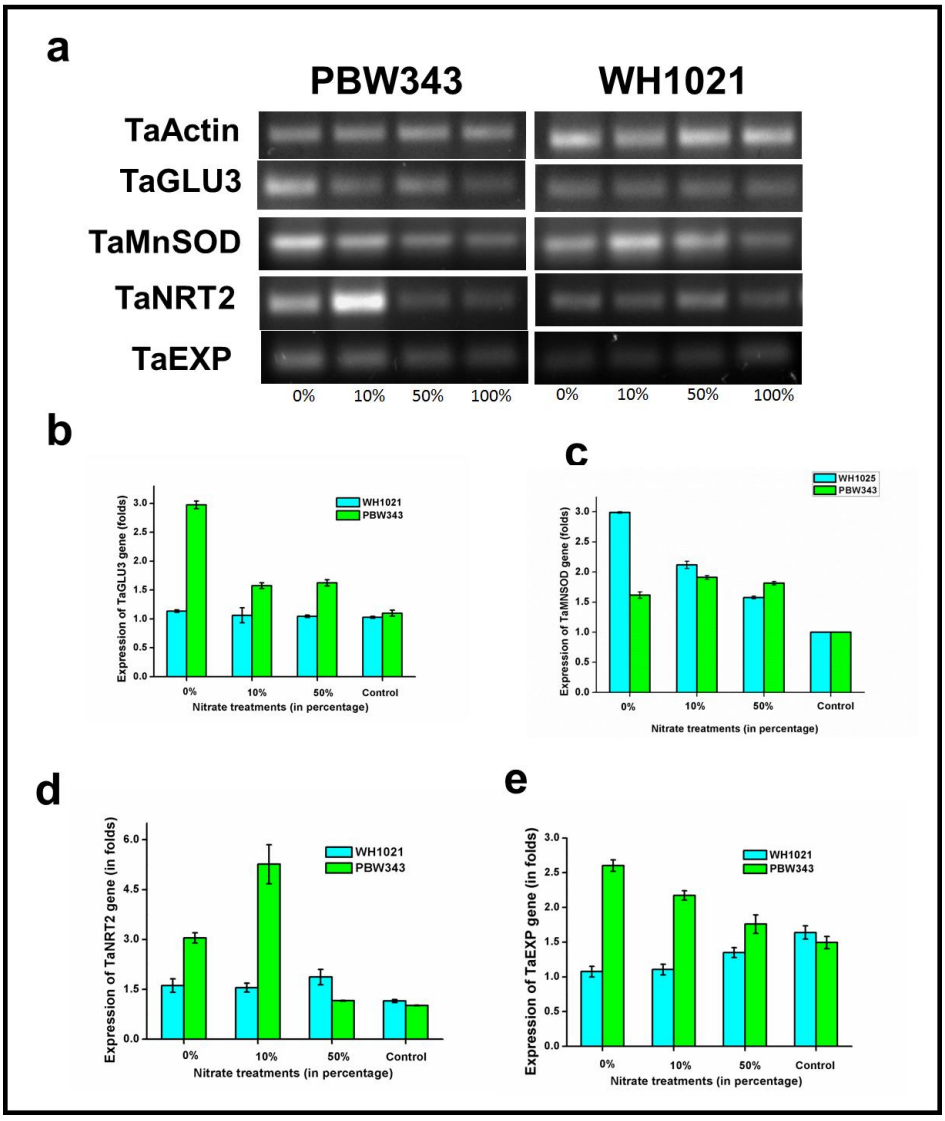

Fig. 4.6. Expression of root architecture related genes in PBW343 and WH1021 wheat cultivar roots. a) agarose gel (1.5 %) showing the RT-PCR results of all the genes along with control in both the wheat cultivars, b) histograms showing the relative expression of *TaGLU3,* c) *TaMNSOD,* d) *TaNRT2* and e) *TaEXP* genes in WH1021 (tolerant) and PBW343 (sensitive) cultivars of wheat at different nitrate concentrations in roots. Nitrate concentration (5 mM) was used as 100 % in this experiment .Values represent the mean ± SD (in 3 replicates).

The results of tBLASTn comparison identified 28 wheat ESTs of which HX148113 was identified to be orthologous of *ZmRTCS* showing 88 % query coverage. The BLASTn search for "draft assembly of gene rich regions" of the 5X database showed 4 contigs for HX148113. These contigs were assembled into a full length *TaRTCS* sequence and intron-exon boundaries were marked by comparing with the genomic and cDNA sequences of *OsRTCS* (closest to wheat). The nucleotide sequences of *ZmRTCS* and its orthologs have been shown in Appendix.

4.6. Structure of the *RTCS* gene

The genomic orthologs from studied species were analyzed for their structure in comparison to the *ZmRTCS* gene. The genomic sequence of *Capsella* was the largest and that of *Panicum* was the smallest (Fig. 4.7.). In monocots, the largest genomic sequence was present in *Oryza* and in dicots, the smallest sequence was present in *Solanum lycospermicum*. The genomic sequence of the *RTCS* gene in all the species consisted of 2 exons (Fig. 4.7.). Interestingly, all the *RTCS* genes showed structurally only one intron. The gene length variation between monocots and dicots was mainly due to the differences in size of introns and insertions in exon 2 (Fig. 4.7.). The monocots showed consistent conservation in between the two exons but dicots showed a drastic change in exon 2 as shown from the intensity of colors in Fig. 4.7. In comparison to the exons of *ZmRTCS* gene, the percent identity in monocots showed more than 90 % similarity of exon 1 while more than 70 % similarity was observed in exon 2. However, the similarity index was comparatively less in both the exons among dicots. The GC content of introns was also lower (20-30 %) in dicots as compared to monocots (50-70 %).

The structural and positional information of the true orthologs of *RTCS* gene in various monocots and dicots is presented in Table 4.6. The results revealed that predicted CDS of *RTCS* gene in all the species ranged from 714 bp-837 bp (Table 4.6.; see representative Fig. 4.7.). The *Glycine max RTCS* ortholog was found to have maximum gene length among all dicots and monocots including wheat (Table 4.6.). The percent similarity of all the *RTCS* orthologs obtained by tBLASTn tool using the sequence of maize *RTCS* gene as a reference were shown in Table 4.7. Among monocots, the query coverage of the *RTCS* gene was more than 85 % and sequence similarity lied between 70-92 %. However, in dicots, the query coverage was near about 50 % while the percent similarity was more than 60 % (Table 4.7.).

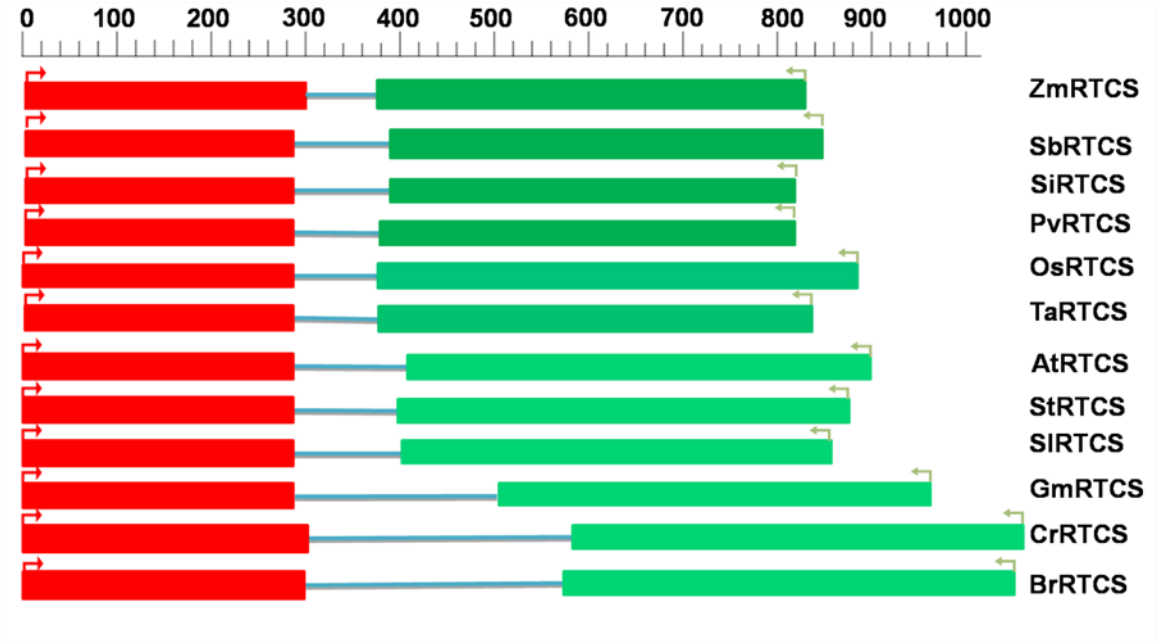

Fig. 4.7. Structure of maize (*Zea mays) RTCS* gene (cDNA) and its orthologs in five monocot (*Sorghum bicolour, Setaria italica, Panicum virgatum, Oryza sativa* and *Triticum aestivum*) and six dicot (*Arabidopsis thaliana, Solanum tuberosum, Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) species of plants. The exons, introns, transcription start site and transcription stop site have been shown by boxes (colored), lines, forward arrows and call out signs, respectively. The intensity of the color in a box represents the nucleotide sequence similarity of a particular species with *Z. mays*.

4.7. Analyses of predicted RTCS ortholog amino acid sequences

The predicted protein sequence of the RTCS ranged from 237 aa (in *Panicum* and *Glycine*) to 278 aa (in *Capsella*). Overall, the predicted ZmRTCS protein sequence showed aa sequence identity of 95 % with *Sorghum*, 73 % with wheat, 79 % with rice and approximately 50 % with all the dicots. Amino acid sequences of six monocots and six dicots were compared in similarity plots to study the conservation of the RTCS protein across monocots and dicots (Fig. 4.8.). The results indicated that approximately first 110 amino acids were conserved in both the clades (Fig. 4.8.).

Table 4.6. Structural and positional characteristics of *RTCS* gene in six monocot and six dicot plant species.

S. No.	Gene symbol	Plant Species	Chromosome number	Gene name /locus name	Database used	Number of introns	Gene length	Length of Coding sequence	No. of Amino acids coded by gene
1	*ZmRTCS*	*Zea Mays*	1	GRMZM2G092542	Maizegdb	1	1245	735	245
2	*SbRTCS*	*Sorghum bicolor*	1	Sb01g046990	Phytozome	1	844	738	244
3	*SiRTCS*	*Seratia Italica*	Scaffold 9	Si040019m.g	Phytozome	1	1248	717	238
4	*PvRTCS*	*Panicum virgatum*	9a	Pavir.Ia04220	Phytozome	1	813	714	237
5	*OsRTCS*	*Oryza Sativa*	3	LOC_Os3g05510	RAPDB	1	1122	780	259
6	*TaRTCS*	*Triticum aestivum*	4	TaRTCS	IWSGC/ URGI	1	918	729	242
7	*AtRTCS*	*Arabidopsis thaliana*	4	AT2G42440.1	TAIR	1	1117	735	244
8	*StRTCS*	*Solanum tuberosum*	1	PGSC0003DMG400022454	Phytozome	1	1148	738	245
9	*SlRTCS*	*Solanum lycopersicum*	9	Solyc09g066270.2	NCBI	1	975	735	244
10	*GmRTCS*	*Glycine max*	13	Glyma.13G121400	Phytozome	1	1275	714	237
11	*CrRTCS*	*Capsella rubella*	Scaffold 3	Carubv10015417	Phytozome	1	1040	837	278
12	*BrRTCS*	*Brassica rapa*	5	Brara.E00288	Phytozome	1	1034	735	244

Table 4.7. Percent similarity comparison of the *RTCS* gene of six monocots and six dicots on the basis of tBLASTn using maize *RTCS* as a reference sequence

Monocots		Dicots	
Gene symbol	**Percent similarity with maize RTCS gene**	**Gene symbol**	**Percent similarity with maize RTCS gene**
SbRTCS	92%	AtRTCS	73%
SiRTCS	86%	StRTCS	73%
OsRTCS	71%	SlRTCS	75%
PvRTCS	89%	GmRTCS	71%
TaRTCS	70%	CrRTCS	60%
		BrRTCS	76%

The conserved domain database analysis of the RTCS consensus sequence was performed as described in Materials and Methods section to determine the functional domains and motifs of the protein. The results showed that the RTCS protein carried an LOB domain of 109 amino acids. This domain was present in all monocots as well as in dicots (Fig. 4.9.). This domain was found to be further subdivided into 3 functional motifs i.e C-motif, GAS-motif and leucine zipper-like motif. The C-motif was found to be conserved in all the monocots except at position 13 in wheat where valine is replaced by alanine. The RRK pattern in C-motif was found to be conserved among both monocots and dicots (Fig. 4.9.). At position 74 of leucine-zipper motif, valine was replaced by isoleucine in wheat from rest of the monocots, however, vice versa was seen in case of *Solanum tuberosum*. The pattern of hydrophobic residues among monocots and dicots was almost same (Fig. 4.10.). Among monocots, arginine (hydrophilic aa) was conserved at position 14 whereas it was replaced by a hydrophobic aa serine in dicots. Similarly, at 24th position of the domain, histidine among monocots was replaced by serine in most dicots. In addition, motif position from 46-48 was also seen with similar differences where less hydrophobic aa (alanine) in monocots is replaced by more hydrophobic aa (leucine) in most dicots. The hydrophobic residues (valine, leucine and isoleucine) in leucine-zipper motif were

conserved at position 70, 74, 78, 81 and 84 among all the plant species. At position 74 in wheat, valine was replaced by isoleucine.

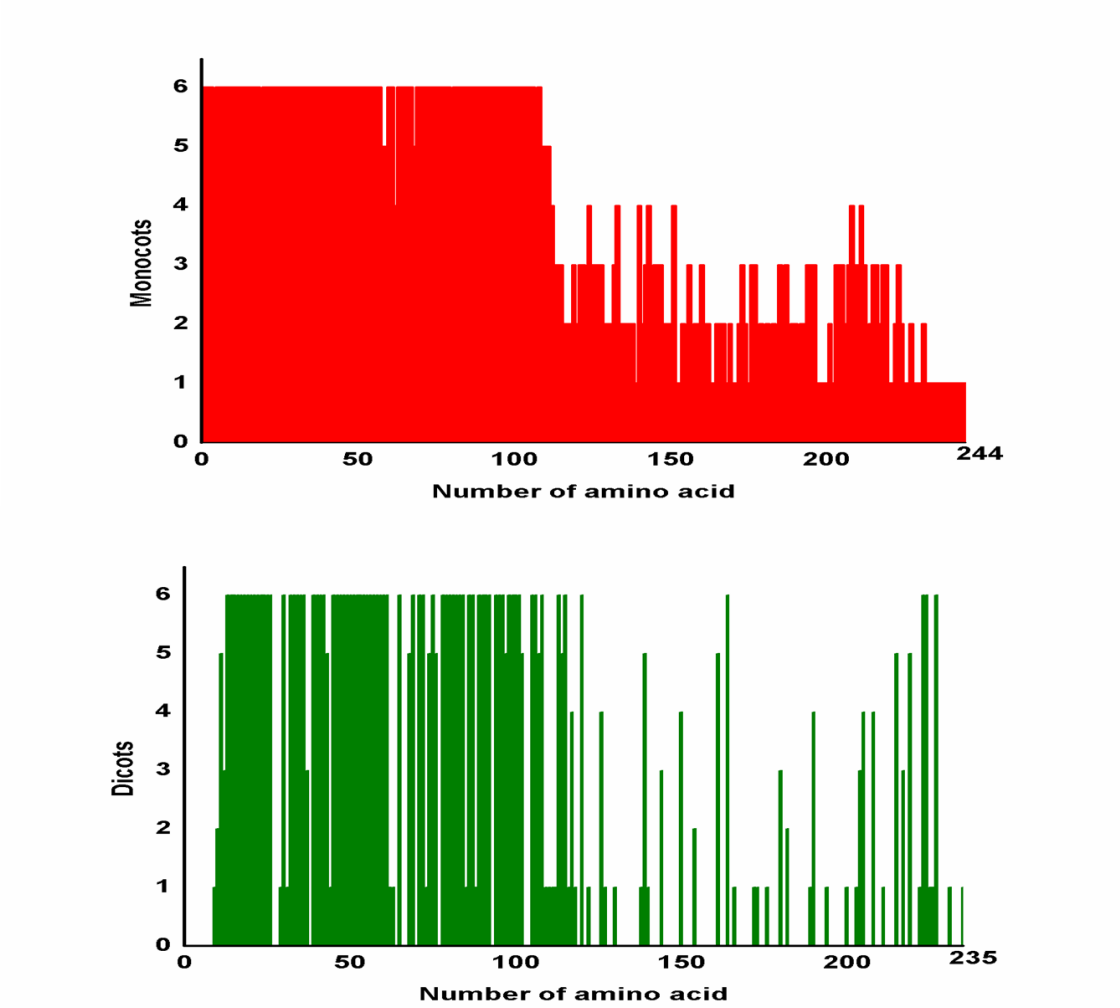

Fig. 4.8. Amino acid conservation in RTCS proteins of six monocot (*Zea mays, Sorghum bicolour, Setaria italica, Oryza sativa, Panicum virgatum* and *Triticum aestivum*,) and six dicot (*Arabidopsis thaliana, Solanum tuberosum, Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) plant species. The presence of similar amino acid against consensus sequence is plotted on a scale of 0-6 for both monocots and dicots where 6 indicates complete amino acid similarity at a particular position and 0 indicates no similarity.

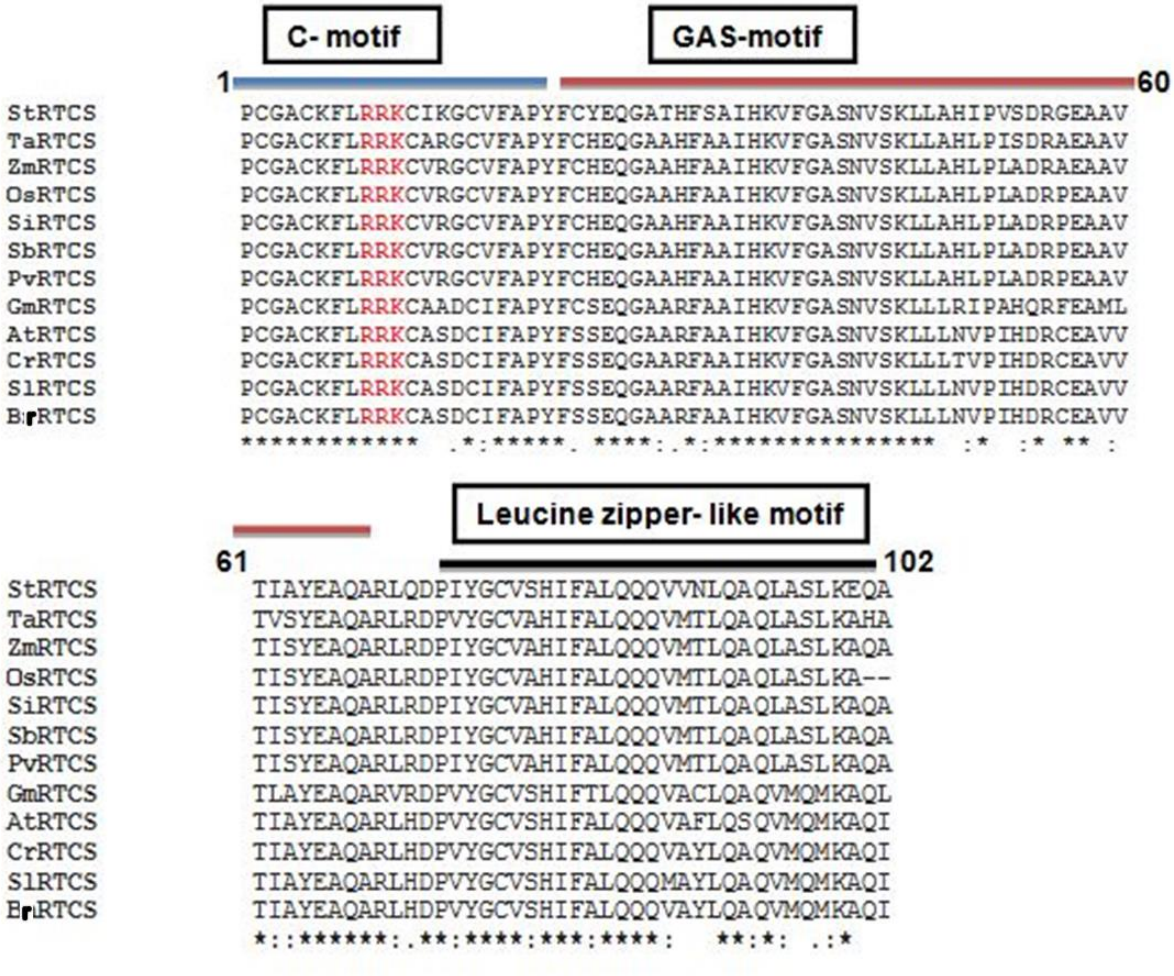

Fig. 4.9. Conservation of motifs in the LOB domains of RTCS proteins of six monocot (*Zea mays, Sorghum bicolour, Setaria italica, Oryza sativa, Panicum virgatum* and *Triticum aestivum*) and six dicot (*Arabidopsis thaliana, Solanum tuberosum, Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) plant species. RRK motif is marked.

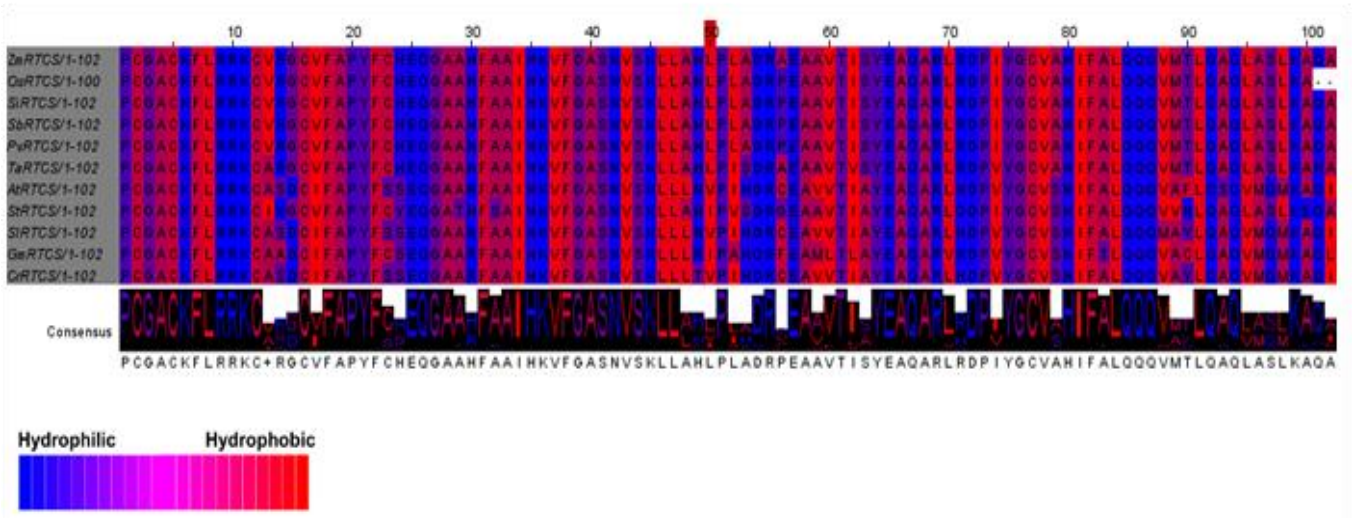

Fig. 4.10. Hydrophobicity conservation in the LOB domains of RTCS proteins in six monocots and six dicots. Except *Oryza,* all the species contain LOB domain of 102 amino acids. Red and blue colors represent hydrophobicity and hydrophilicity, respectively. The extent of hydrophobicity has been represented by the intensity of the color.

4.8. Molecular Modeling of RTCS Protein

The protein blast of ZmRTCS was done using BLASTp tool. Homology modeling of ZmRTCS was not possible because the matching templates had very high E-value and less query coverage. So, the 3D structure was generated by iTASSER tool and visualized. The five models generated from iTASSER were subjected to SAVES server for structure analysis. The same procedure was done for TaRTCS. Refined model structures of both ZmRTCS and TaRTCS have been shown in Fig. 4.11. The TaRTCS model was further evaluated for the stereo-chemical quality by PROCHECK and results showed more than 89 % residues of predicted structure of TaRTCS protein in allowed region (Fig. 4.12-a). The validation of modeled structure of TaRTCS protein was done by using server ProSA-web. This analysis showed that the protein folding energy was negative and z-score value (-3.9) was in the range of native conformation (Fig. 4.12-b). Hence the overall model quality of TaRTCS protein was found good for further analysis.

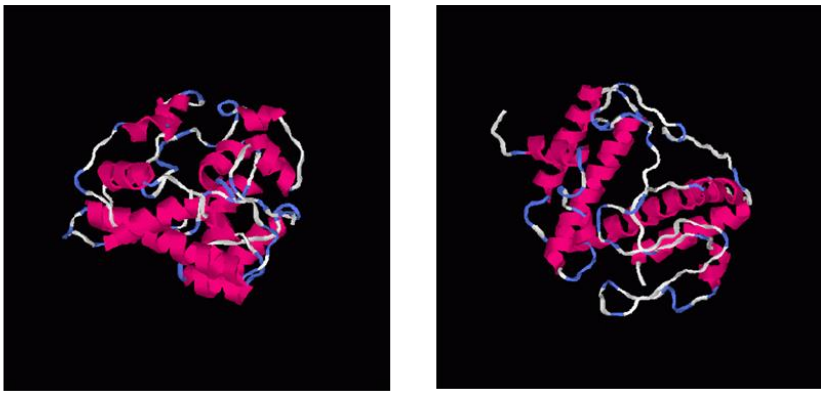

Fig. 4.11. Predicted 3D-modeled structure of RTCS protein by Scratch Protein Predictor tool in maize and wheat. These results were viewed using PyMOL.

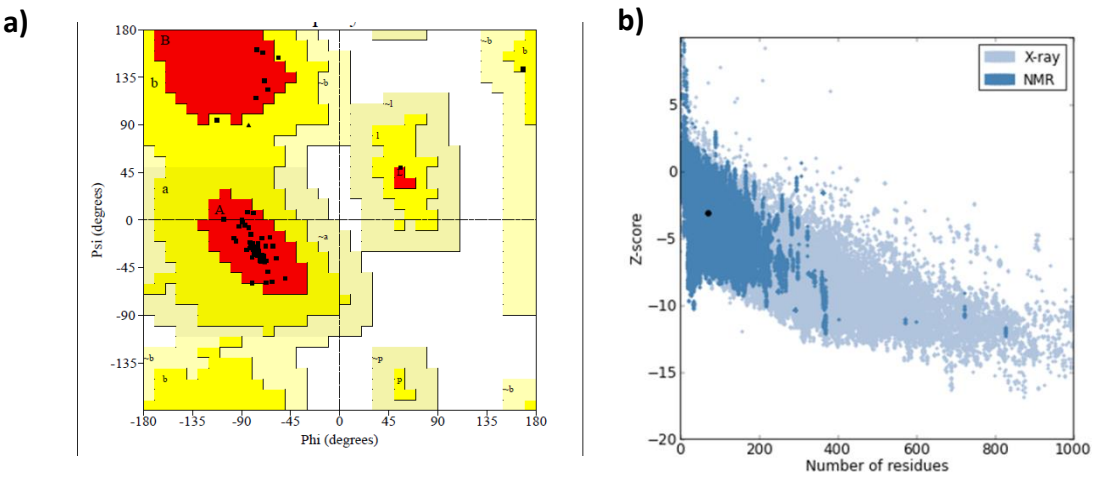

Fig. 4.12. Validation of predicted model of TaRTCS protein (a) Ramachandran plot of predicted model of TaRTCS: This figure is generated by PROCHECK. The red regions in the graph indicate the most allowed or preferred regions covering 89 %, whereas the yellow regions represent allowed regions covering 9 % of the aa residues, glycine is represented by triangles and other residues are represented by squares and (b) result of predicted model by PROSA-web where *z*-scores of all protein chains in PDB is determined by X-ray crystallography (light blue) or NMR spectroscopy (dark blue).

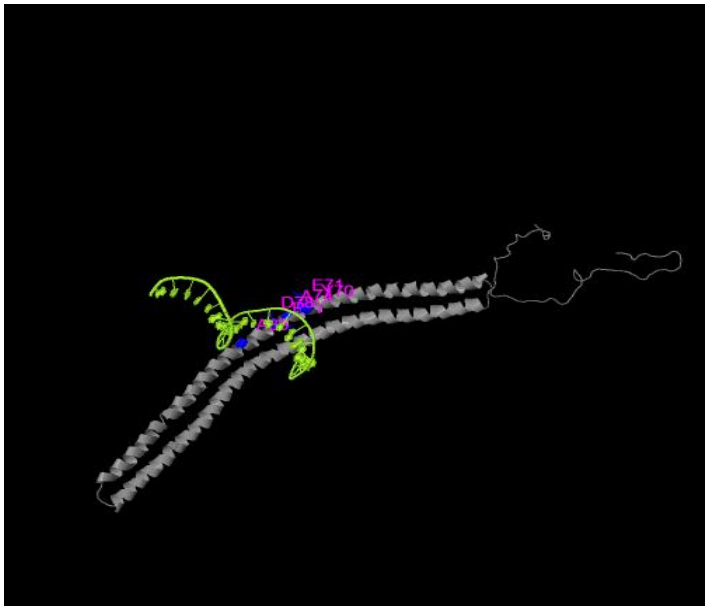

Fig. 4.13. Predicted ligand binding interactions of leucine-zipper like-motif in TaRTCS protein at positions 70, 71, 74, 77, 78 and 85 with nucleic acid (Viewed by PyMOL)

The ligand binding analysis was performed to predict the binding pattern of various ligands to TaRTCS. The results indicated the binding of DNA helix to the leucine zipper motif present in TaRTCS protein as shown in Fig. 4.13. The aa residues at positions 70, 71,74,77,78 and 85 were predicted to be involved in this binding.

4.9. Phylogenetic analysis of *RTCS* gene

The total sequence alignment was used to compute an unrooted phylogenetics tree showing evolutionary divergence of TaRTCS and 11 other species (Fig. 4.14.). All the bootstrapped values are summarized in the consensus tree based on a majority rule. Each node is labeled with bootstrap values that are the percentage of appearance of a particular clade. As seen in the Fig. 4.14., all species were divided into two major clades. However in monocots, three subgroups were formed. One group represents *Z*ea *mays* and *Sorghum bicolor* with close relativeness to *Setaria italica* and *Panicum virgatum.* Rice and wheat have been shown separately in the phylogram. Most of the dicots appears in a separate clade from monocots with St*RTCS* being an exception. While At*RTCS* and Gm*RTCS* were in a group of their own, their node branch lengths showed relatively long divergence (Fig. 4.14.).

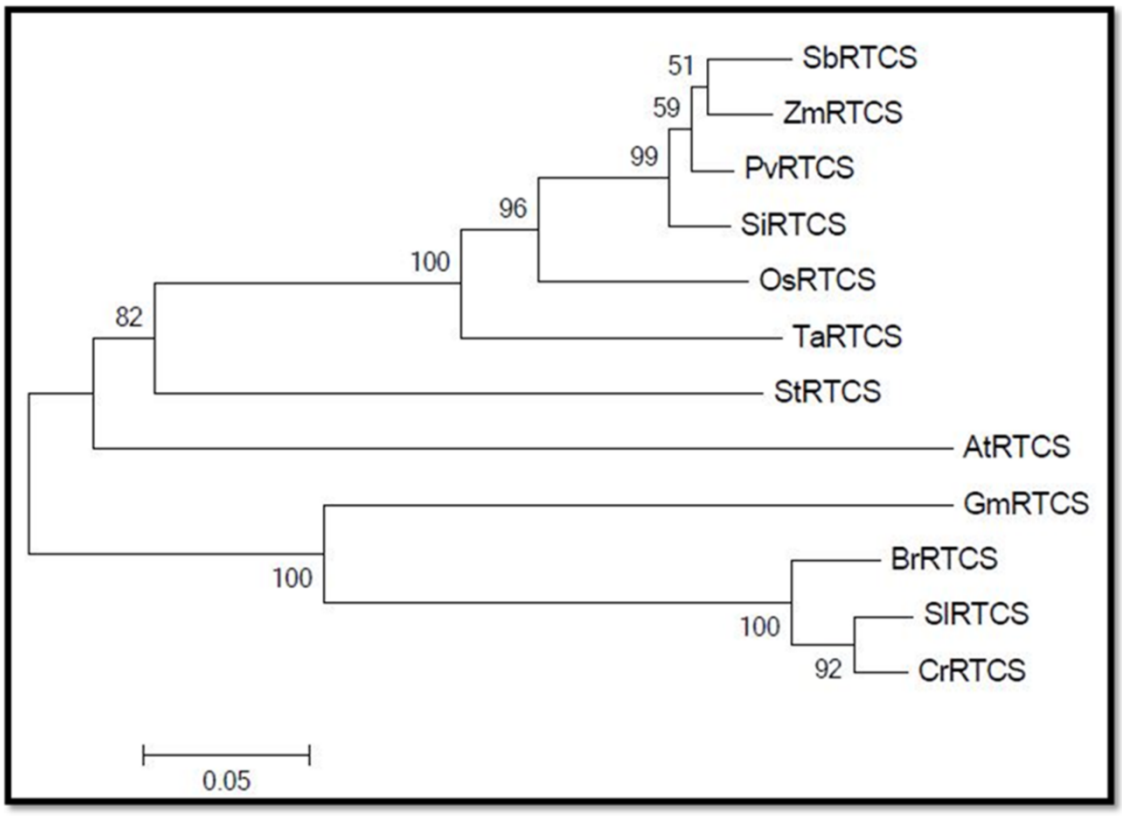

Fig. 4.14. The unrooted phylogenetic tree showing the evolutionary divergence of *RTCS* in six monocot (*Zea mays, Sorghum bicolour, Setaria italica, Oryza sativa, Panicum virgatum* and *Triticum aestivum*) and six dicot (*Arabidopsis thaliana, Solanum tuberosum, Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) plant species. Bootstrap values are indicated on the roots.

In all identified orthologs of *RTCS* gene, the average of statistics π (Nei, 1987) and θ (Watterson, 1975) were 0.70015 and 0.90423, respectively, as shown in Table 4.8. The π values for monocots and dicots were 0.54351 and 0.71245, respectively. Calculated Tajima's D statistics for all homologues showed a value of 1.0971, which was negative but not significant even at $p > 0.10$. All monocot genomic DNAs of *RTCS* carried CpG islands at both 5' and 3' ends and even the whole gene structure in monocots falls under CpG islands. Interestingly, none of the dicots carried a single CpG island (Fig.4.15.).

Table 4.8. Summary of the sequence polymorphism of the *RTCS* gene and test statistics in six monocot (*Zea mays, Sorghum bicolour, Setaria italica, Oryza sativa, Panicum virgatum* and *Triticum aestivum*) and six dicot (*Arabidopsis thaliana, Solanum tuberosum, Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) plant species.

Clades	No. of Haplotypes	Theta (θ)	Pi(π)	D(Tajima)	D*	F*
Monocots	6	0.66614	0.54351	1.1994	0.4568	0.6777
Dicots	6	0.9117	0.71245	1.672	0.5109	0.811
Total		**0.90423**	**0.70015**	**-1.0971**		

Abbreviations: θ- number of segregating sites per site between two sequences, π- average number of nucleotide differences per site between two sequences, D-Tajima's estimate D test, D*- Fu and Li D* test, F* -Fu and Li F* test

Fig. 4.15. CpG islands detection in the *RTCS* gene in six monocot (*Zea mays, Sorghum bicolour, Setaria italica, Oryza sativa, Panicum virgatum* and *Triticum aestivum*) and six dicot (*Arabidopsis thaliana, Solanum tuberosum, Solanum lycopersicum, Glycine max, Capsella rubella* and *Brassica rapa*) plant species. Marked area indicates the presence of CpG islands.

4.10. Physical mapping of *RTCS* gene in wheat

In-silico analysis showed that each of the three-wheat chromosome 4AL, 4BL and 4DL carried an ortholog of maize *RTCS* gene, indicating the presence of atleast 3 copies of *RTCS* gene in wheat. The Ta*RTCS* gene was not amplified in 4A, 4B and 4D chromosome deletion lines, which confirmed the location of this gene (Fig. 4.16.).

4.11. Level of expression of *TaRTCS* gene in different tissues of wheat plant

The expression of Ta*RTCS* gene was studied at different stages of wheat, results have been presented in Fig. 4.17. The gene was expressed in all the vegetative stage of wheat development ranging from early germination to flag leaf developmental stage. The expression of Ta*RTCS* was found to be highest in seedling roots (6.5 folds normalized to actin) followed by germinating roots (5 folds) and crown (3.8 folds). The expression in coleoptile and shoots was found to be relatively lower than that in roots (Fig. 4.17-b). The sequences of the designed primers along with their amplification sizes and optimized annealing temperatures have been presented in Table 4.9.

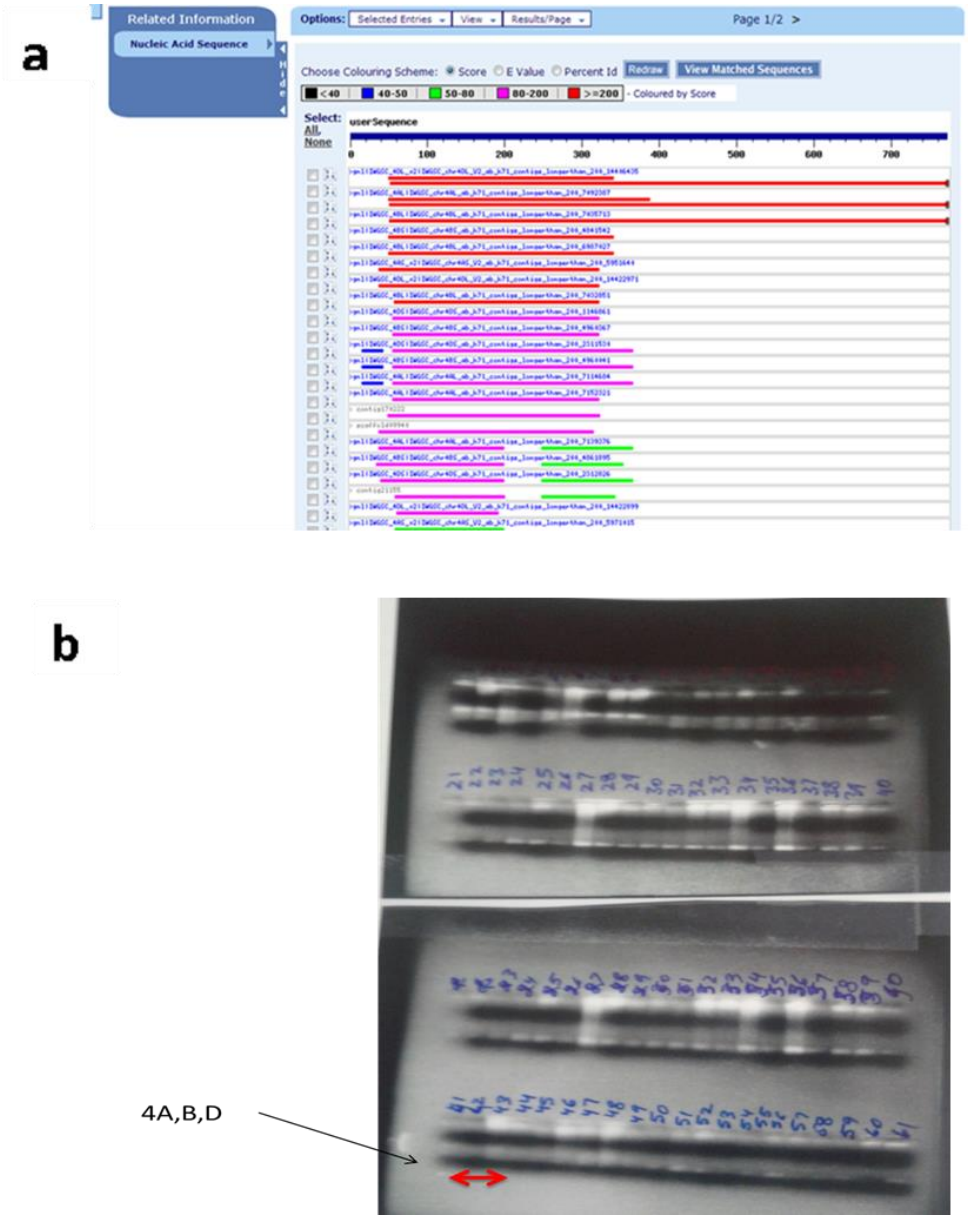

Fig. 4.16. Physical locations of *RTCS* gene in wheat. (a) the location of the *RTCS* gene on chromosome 4A, 4B and 4D given by IWSGC and (b) agarose gel (1.2 %) showing the PCR amplification of *RTCS* gene in deletion lines of wheat. No band was present in deletion line number 41, 42 and 43.

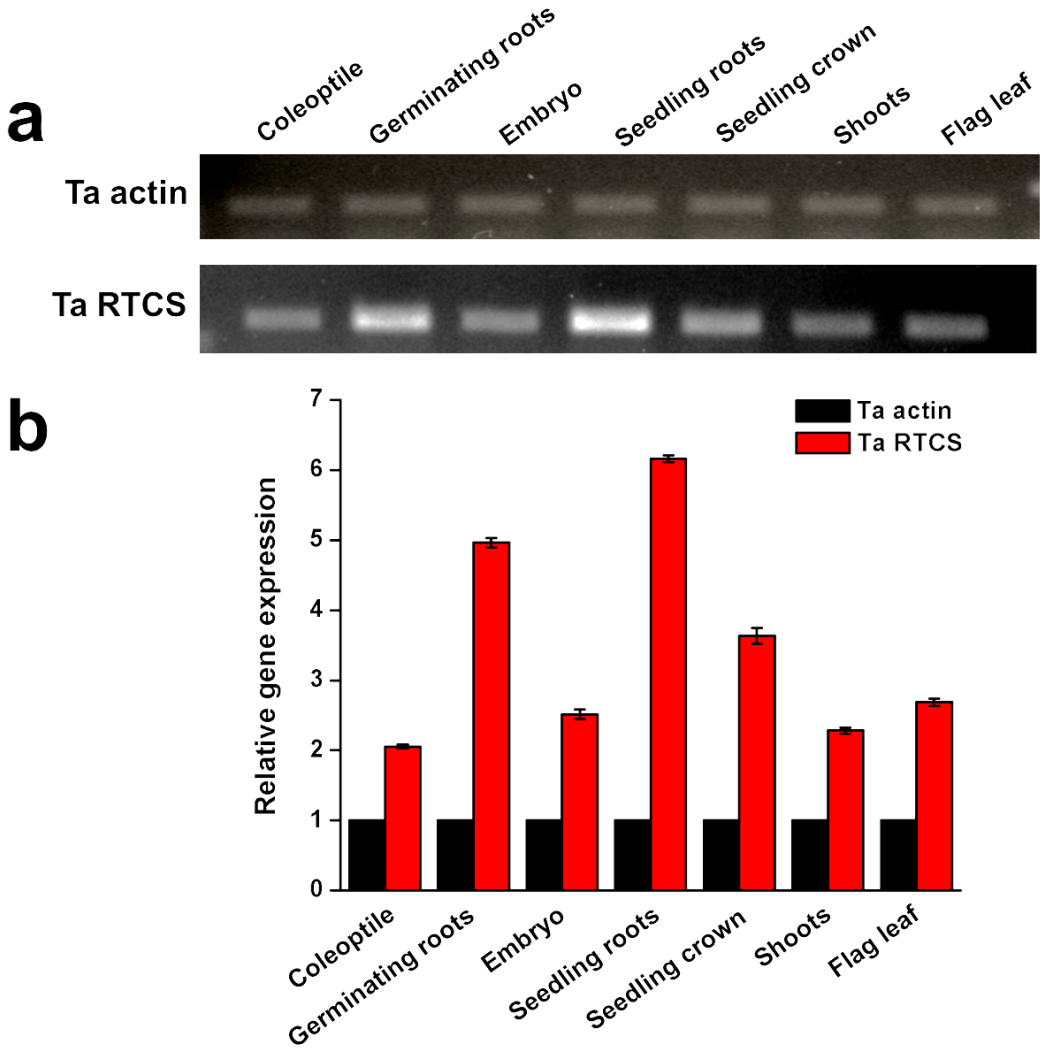

Fig. 4.17. Expression of *TaRTCS* gene in different tissues of wheat plants (a) agarose gel (1.5 %) picture of DNA bands obtained by amplification with primers of *TaRTCS* gene from cDNAs of different tissues of wheat. Actin was used as an internal control and (b) histogram showing the semi-quantitative expression of *TaRTCS* gene relative to the expression of actin gene. The error bars represent the standard deviations of the means (n=5).

4.12. Virus induced gene silencing (VIGS)

4.12.1. Construction of VIGS construct with *TaRTCS* gene insert

On the basis of *in-silico* prediction of wheat orthologous sequences, antisense oligo specific for *RTCS* gene was designed and ligated in BSMV gamma plasmid vector as described in Material and Method section. The vector map of BSMV plasmid having *TaRTCS* insert is shown in Fig. 4.18. The recombinant plasmid was introduced into *E.coli* DH5α cells by transformation. The ampicillin resistant (Amp^R) transformants were selected (Fig. 4.19.) and the recombinant plasmid construction was confirmed by colony PCR (Fig. 4.20.), restriction analysis with *Not*I and *Pac*I restriction enzymes (Fig. 4.21.) and DNA sequencing (Fig. 4.22.). The pα, pβ and the target specific pγ plasmids, linearized with restriction enzymes *Mlu*I, *Spe*I and *BSSH*I, respectively, are shown in Fig.4.23. These linear plasmids were diluted to a concentration of 125 ng/µl (Fig. 4.24.). The diluted plasmids were used for *in-vitro* transcription reaction. Fig. 4.25. shows the RNA bands obtained after *in-vitro* transcription of each linearized plasmid. The RNA of each plasmid was mixed in equal amounts to form a complete live BSMV virus to infect wheat plants.

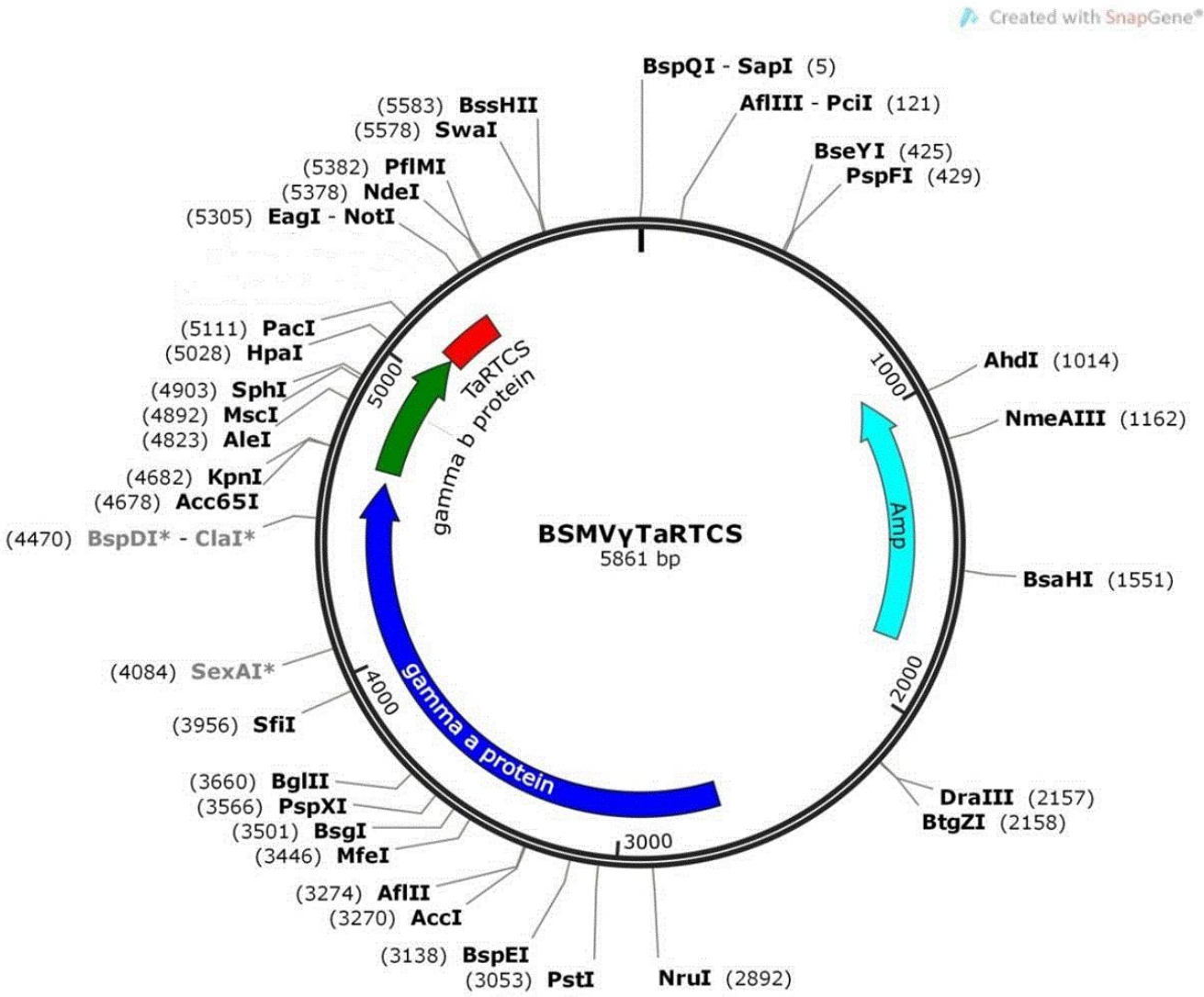

Fig. 4.18. Cloned construct of Ta*RTCS* unique gene sequence in empty γ*PDS* of BSMV cloning vector named as γTa*RTCS*.

4.12.2. Optimization of control and marker genes

The procedure for growth and inoculation of construct on wheat plants has been shown in Fig. 4.26. The barley phytoenedesaturase (b*PDS*) gene, which is required for the synthesis of carotenoids (compounds that protect chlorophyll from loss of chlorophyll pigmentation), was used as a visual marker to assess RNA silencing effect. In all experimental seedlings (uninoculated and inoculated), the infection of virus was determined by PCR analysis with primers designed from *Pac*I-*Not*I flanking sites of gamma genome of BSMV (Fig. 4.27-a). The sequences of the designed primers along with their amplification sizes and optimized annealing temperatures have been presented in Table 4.9.

The wheat seedlings inoculated with MCS (negative control) virus and virus carrying PDS silencing construct showed 100 % incidence of virus infection (percentage of plants showing viral infection), while FES treated (without virus) seedlings showed 0 % incidence of virus infection (Fig. 4.27-b). The wheat seedlings inoculated with γ.bPDS4 exhibited intense photo-bleaching (white streaks) in most of the leaf area. The extent and intensity of photo-bleaching and severity of viral symptoms (necrosis, leaf curling and stunting) varied among the inoculated plants. In 10-day-old (2 leaf stage) inoculated wheat seedlings, photo-bleaching symptoms became visible at 8-10 dpi (days post inoculation) onwards (4 leaf stage) followed by sharp streaks at 21 to 25 dpi. The results of photo-bleaching of leaf are presented in Fig. 4.27-b. It was observed that the symptoms of photo-bleaching declined gradually in the later stages of plant development.

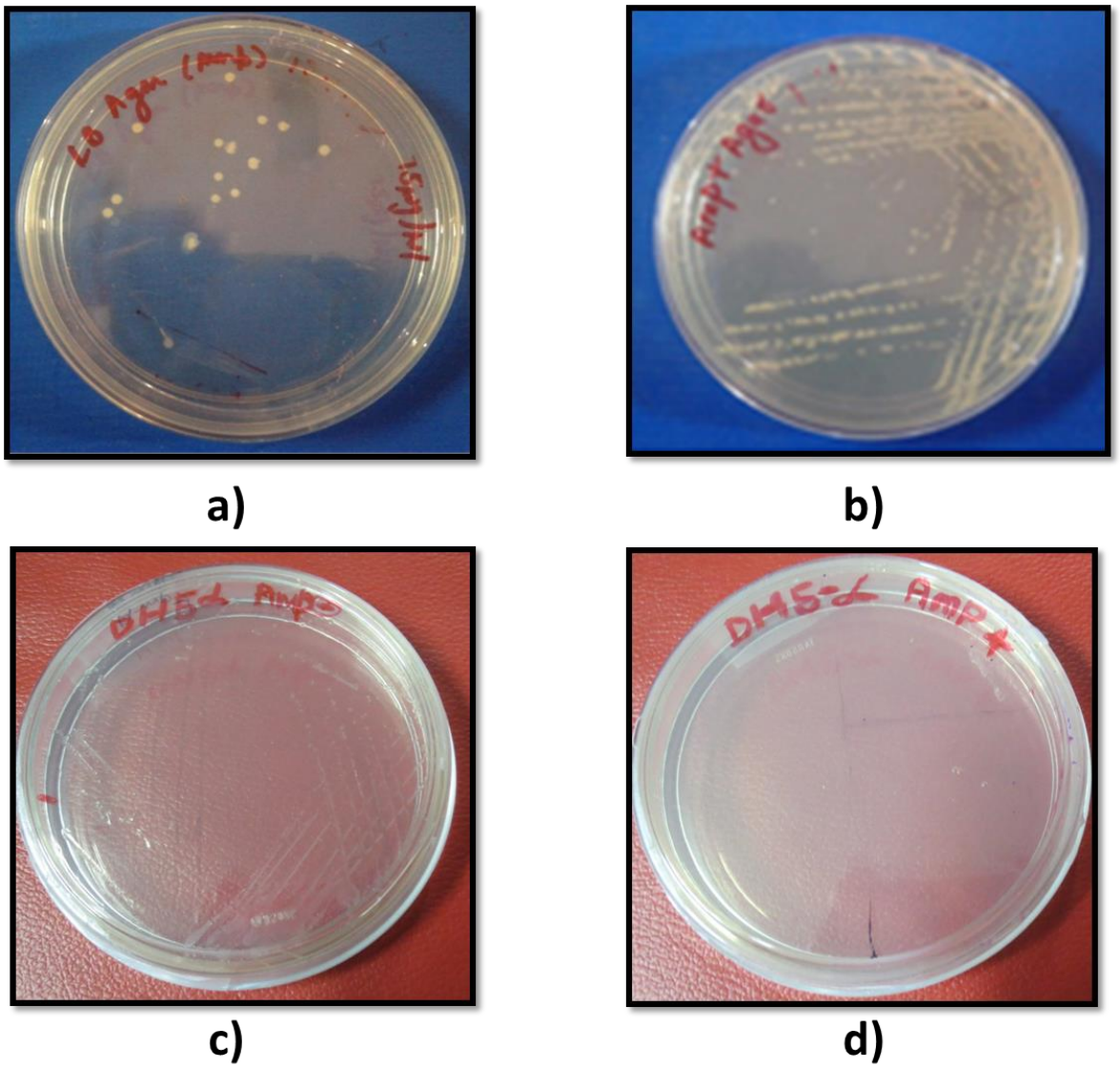

Fig. 4.19. LB solidified medium plate containing 100 µg/ml ampicillin, showing (a) growth of transformed *E. coli* DH5α colonies containing 100 bp *TaRTCS* unique sequence ligated to empty BSMV vector, (b) colonies obtained by streaking the positively transformed colony of ligated *TaRTCS* gene sequence, (c) negative control with no ampicillin and (d) positive control.

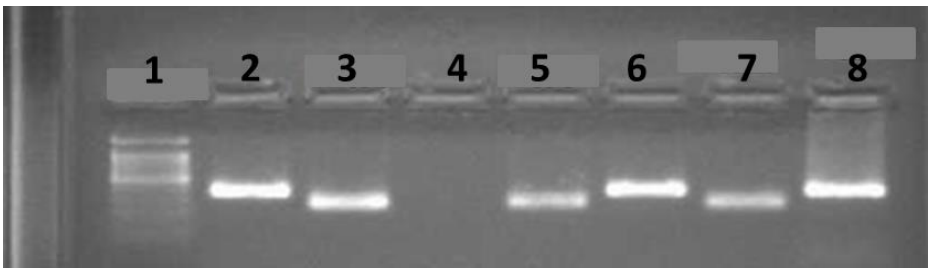

Fig. 4.20. Agarose gel (1 %) picture of DNA bands obtained after PCR amplification with primers specific for pγ plasmid from the transformed colonies. Lane 1- 100 bp DNA ladder, Lanes 3, 5 and 7- amplified 264 bp *TaRTCS* gene from transformed colonies and Lanes 2, 6 and 8- amplified 364 bp *PDS* gene from transformed colonies.

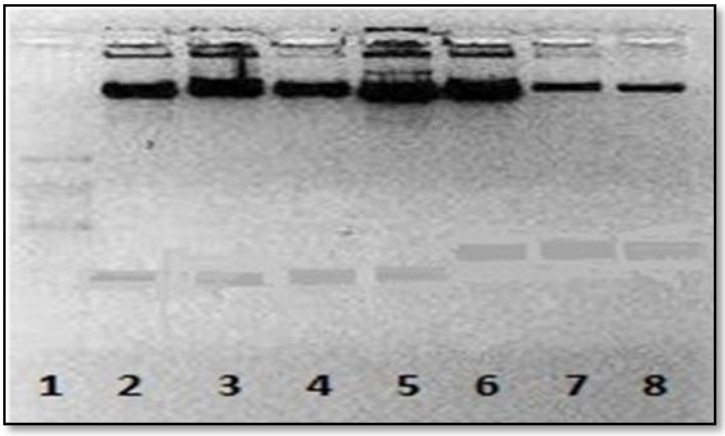

Fig. 4.21. Agarose gel (1 %) picture showing bands obtained after the restriction digestion of plasmids. Lane 1- 100 bp DNA Ladder, Lane 2-5- digested γRTCS plasmids showing the 100 bp insert and Lanes 6-8 digested γPDS plasmids showing the 185 bp insert.

CCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGG
CCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTG
AGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGG
ATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGACGCGCGCGTGGTCTTCCCTTG
GGGGACCGAAGCTGAGCTTCGGCTCAGTATGCACACTCTTAAAGTGTGACGCAGCTACCGGAAGTTGTAGCTTAC
ATACCAATTCCTAAATTTTCTCTCCAGAGTCCGTTAAGATTCATGGTTTCCAATTCAGGCATCGTTTTCAAGTTCGAT
TATAGTGGACTTGCAAACACTCCCATCATATGGTTGATGGGCACCATCAGATTTGAATGATCTGATCAAACATTTTT
TTTTTTTTTTAGCTAGCTGAGCGGCCGC**AGGTGGCCGCCGCCGCGGATCAGCACGACCACCAGTCCATGACGTCC**
ACGCAGAGCAGTCCGCTGCTACAGCGGCGCCACAGCGCTTAATTAATCAGCTAGCAACGGAAGAAGAATCATCA
CATCCAACAGAATCTTCAAAAGAAGAAGCTACGGACTTACGTATTGCGTTAACCTCACTTTCAAGCTTAGCCATTTT
TACGATATGAGAAAGTTTCCAGCCTCCTGCATCCTTCTTCTGGAGAAATTCAAAGAAAGAAACC

Fig. 4.22. Sequence of plasmid DNA containing 100 bp unique fragment of *TaRTCS* gene ligated into cloning vector of γBSMV

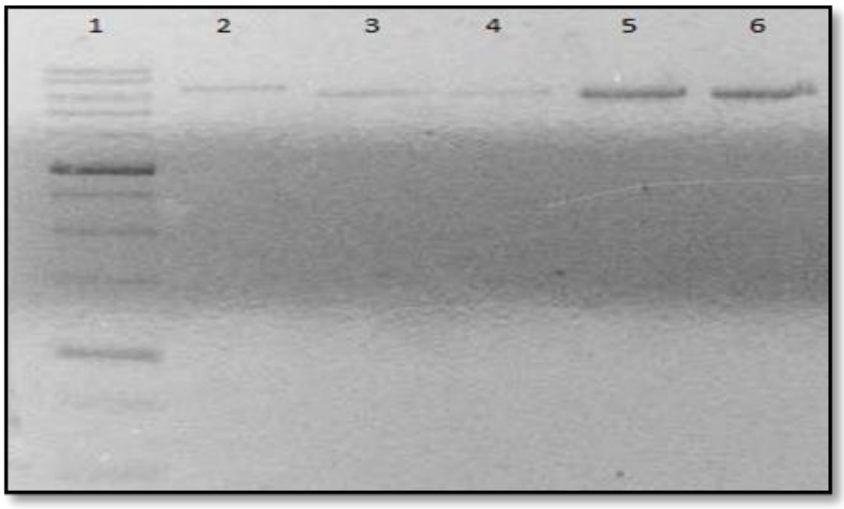

Fig. 4.23. Agarose gel (0.8 %) picture showing the bands obtained by linearization of plasmids using restriction enzymes. Lane 1- 1kb ladder, Lane 2- alpha plasmid, Lane 3- beta plasmid, Lane 4- γMCS plasmid, Lane 5- γPDS and Lane 6- γRTCS.

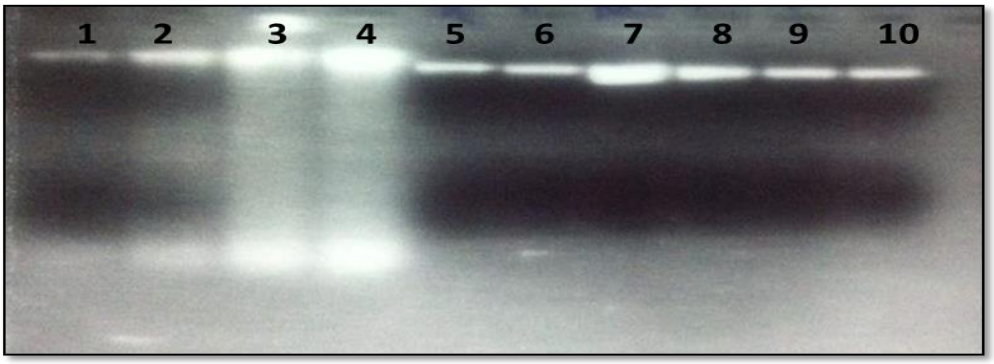

Fig. 4.24. Agarose gel (0.8 %) picture showing the concentration of different diluted linearized plasmids for *in vitro* transcription. Lane1-25 ng/µl, Lane 2-50 ng/µl , Lane 3-100 ng/µl , Lane 4- 200 ng/µl , Lane 5- α plasmid, Lane 6- β plasmid, Lane 7- γMCS plasmid, Lane 8- γPDS plasmid, Lane 9 and 10- γRTCS plasmid.

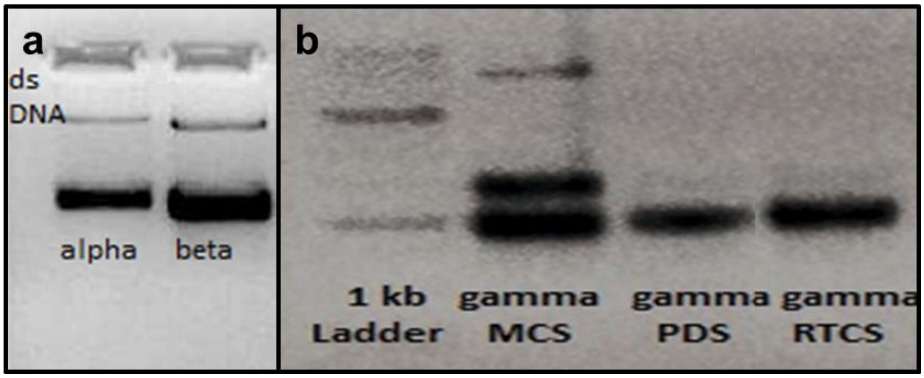

Fig. 4.25. Agarose gel (1 %) picture showing the bands obtained by transcription of plasmids carrying (a) alpha and beta subunits, (b) gamma subunits having *MCS, PDS* and *RTCS* gene inserts.

4.12.3. Silencing of RTCS in root tissues

To determine the function of wheat *RTCS* gene (an orthologue of *Zea mays RTCS*), BSMV-based VIGS was employed to target *TaRTCS* in roots of wheat plants. Fifteen inoculated plants with infectious virus containing γ.TaRTCS resulted in a significantly decreased root length in wheat plants (up to 30 %, when compared with mean root length of control plants) as shown in Table 4.10. and Fig. 4.27-c. Quantitative real-time PCR analysis showed up to 66 % reduction of *RTCS* transcript in roots as compared to γ.TaMCS inoculated plants. The decrease in the transcript level of *PDS* gene was 82 % in γ.TaPDS4 inoculated wheat plants as compared to the uninoculated control plants. The *RTCS* transcript reduction in roots of *RTCS*-silenced plants showed significant positive correlation with seminal root length (Fig. 4.27-c). The phenotypic observations of all plants showed that virus inoculated plants (either only virus or virus carrying gene silencing construct) were weak and less vigorous than either control or FES inoculated plants. No other deleterious effect of the virus on root and shoot development was observed.

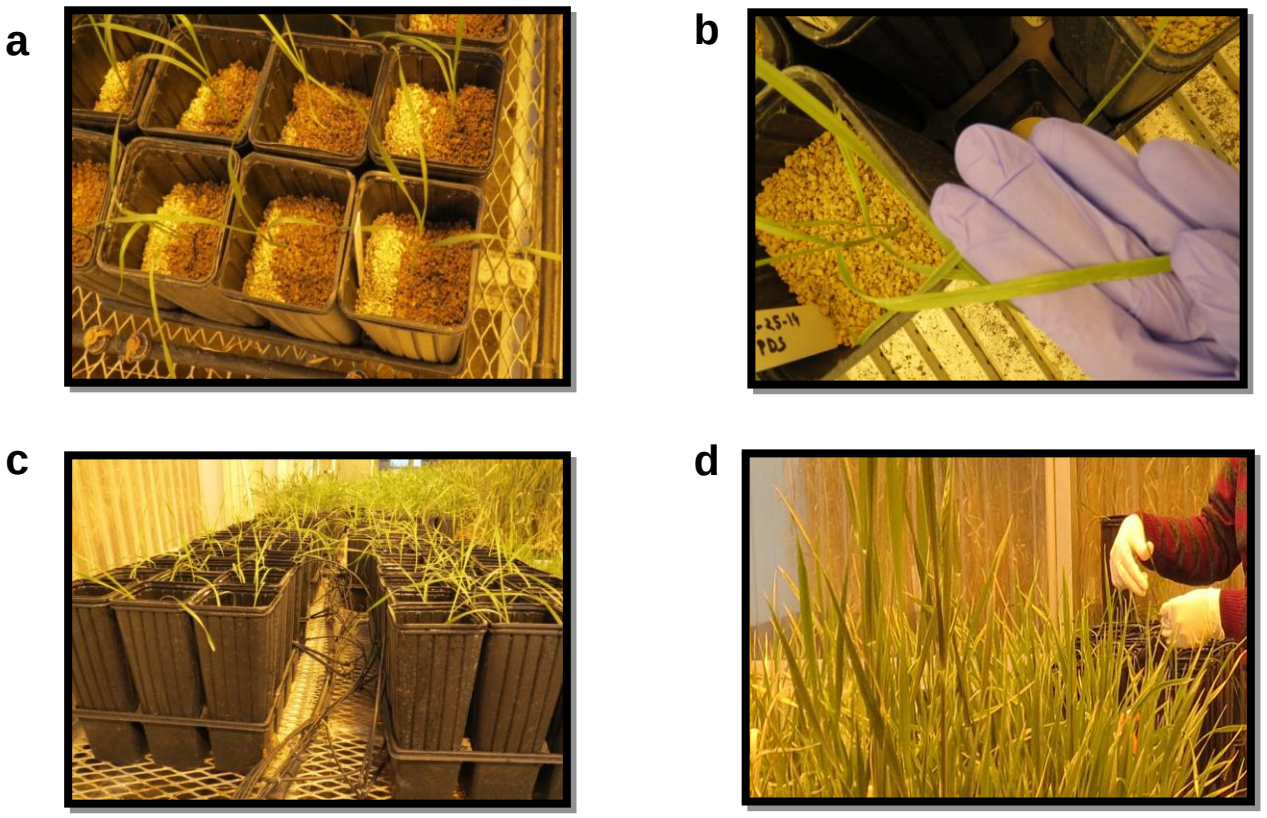

Fig. 4.26. Virus induced gene silencing procedure in Chinese spring wheat plants (a) plants grown in turface MVP material under 16 h of light/8 h of dark regime at 24 ˚C/18 ˚C temperatures, respectively, (b) inoculation of plants with tripartite virus containing gene of interest at two leaf stage, (c) inoculated plants kept under green house for 16 h of light/8 h of dark regime at 24 ˚C/18 ˚C temperatures, respectively, with supply of portable nutrient water on alternate days and (d) collecting the samples of wheat plants after 21 days of inoculation.

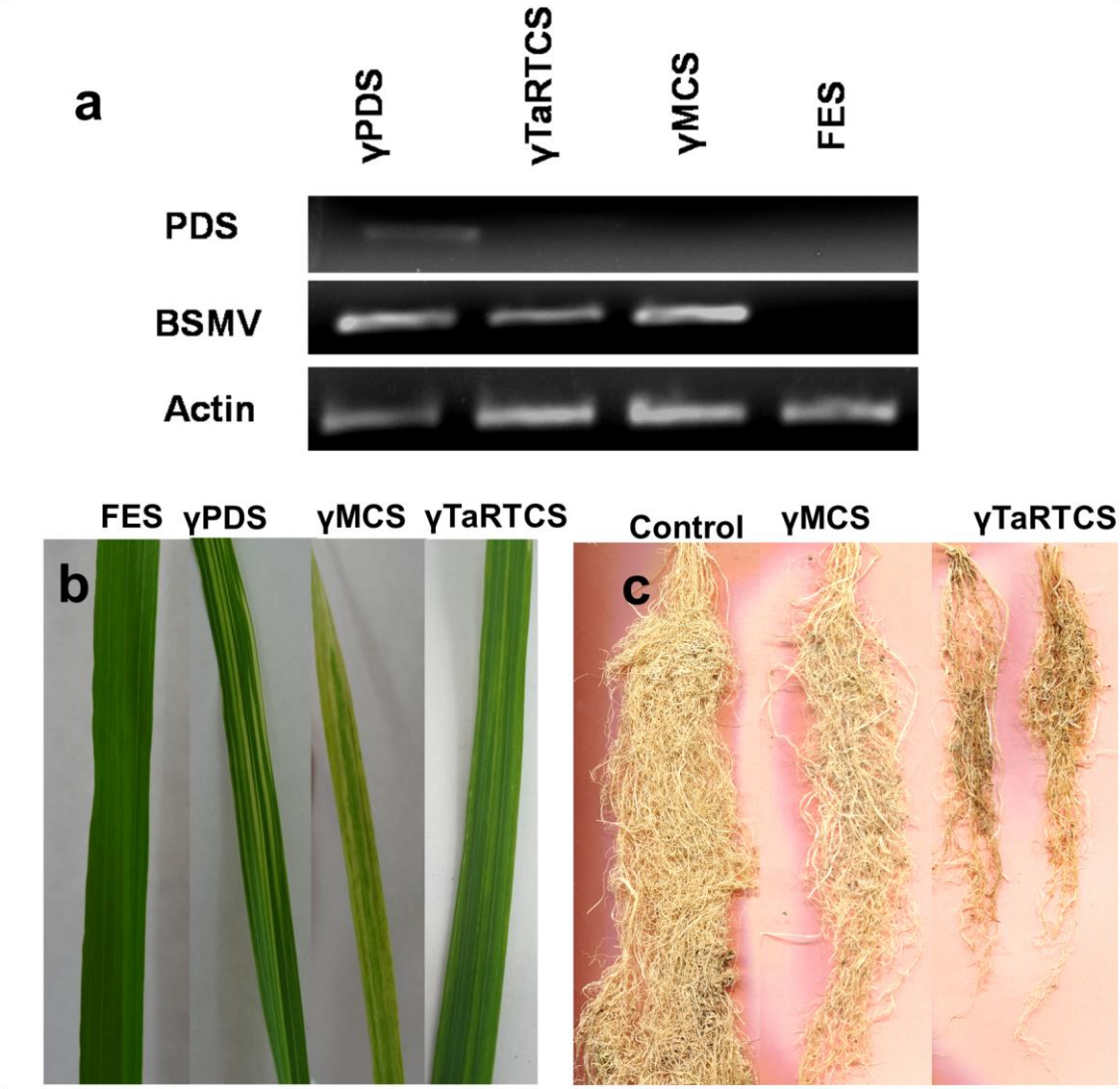

Fig. 4.27. Virus induced silencing of *RTCS*, *PDS* and *MCS* genes of Chinese spring wheat. (a) agarose gel picture of DNA bands obtained by amplification with primers of *PDS* and *BSMV* genes from the cDNAs of different tissues. For the amplification of *PDS* and *BSMV* genes, cDNAs were taken from leaf and both leaf and root tissues, respectively. Actin was used as an internal control, (b) leaves showing the effect of inoculation with γPDS, γMCS and Ta γRTCS constructs and (c) roots showing the effect of inoculation with Ta γRTCS construct.

Table. 4.9. Details of primer sequences and results of amplification of cDNAs regions with various primers at the optimized annealing temperatures

Gene name	**Primer sequence**	**Amplicon length in bp (cDNA)**	**Ta (^{o}C)**
RTCS 1	F 5’ ATCAGCACGACCACCAGTC 3’ R 5’CGAACGAGGAACGAGTGAAT 3’	177	60
RTCS 2	F 5’ AGCAGGCGTGGCAGGTG 3’ R 5’ GTCGGAGCGCTGGTGGG 3’	207	59
RTCS 3	F 5’ TAGTGGTTCTTACGAGCGCT 3’ R 5’CTACATGATGTACCACGCGC 3’	192	58
RTCS 4	F 5’ CTCCCACGTAGCCCTTGAC 3’ R 5’ CAGCAACGTCTCCAAGCTC 3’	242	55
ACTIN 1	F5’TGTGCTTGATTCTGGTGATGGTGTG 3’ R 5’CGATTTCCCGCTCAGCAGTTGT 3’.	247	60
ACTIN 2	F 5’AGGCGGAGTACGATGAGTCT 3’ R 5’ CCCCTTATTCCTCTGAGGGT 3’	163	57
BSMV	F 5’GTGATGATTCTTCTTCCGTTGC3’ R 5’ CGATGCCTGAATTGGAAACC 3’	364	59
PDS	F 5’ CCAAACCGTTCAATGCTGGA 3’ R 5’ TGCACTATGGACTGAGCACA 3’	338	57
IAA	F 5’ATGGTTGAGGCAGTGAACGG 3’ R 5’ AGAACACCATCCTTGTGGCA 3’	237	57

Table. 4.10. Mean root length and transcript abundance in wheat plants silenced with pγ.TaRTCSas, pγ.TaMCSas and pγ.TaPDSas constructs in comparison to control wheat plants

		pγ.TaRTCSas	pγ.TaMCSas	pγ.TaPDSas
	Control Root	TaRTCS	MCS	PDS
Root length (cm)	40±.971a	28±1.08b	36±0.478a	38±0.611a
Gene Expression	1	0.34±0.29a	0.94±0.13c	0.18±0.22b

The error bars represent the standard deviation of mean of 10 plants. Letters indicate significant (p <0.05) difference among the means.

4.13. The effect of IAA on the expression of Ta*RTCS* and Ta*IAA* genes in wheat plants

The real time PCR results of the expression of *TaIAA* and *TaRTCS* genes in the Chinese spring seedlings treated with 10^{-5} mM concentration of IAA are presented in Fig. 4.28. *TaIAA* showed maximum expression after 3 h exposure to IAA. The expression of *TaIAA* showed linear decrease afterwards. At the exposure of 1h, the expression of *TaRTCS* was almost negligible. The expression of *TaRTCS* gene was significantly higher in case of IAA exposure for 6 h (6.2 times). After 24 h of exposure, the expression of both the genes was considerably reduced.

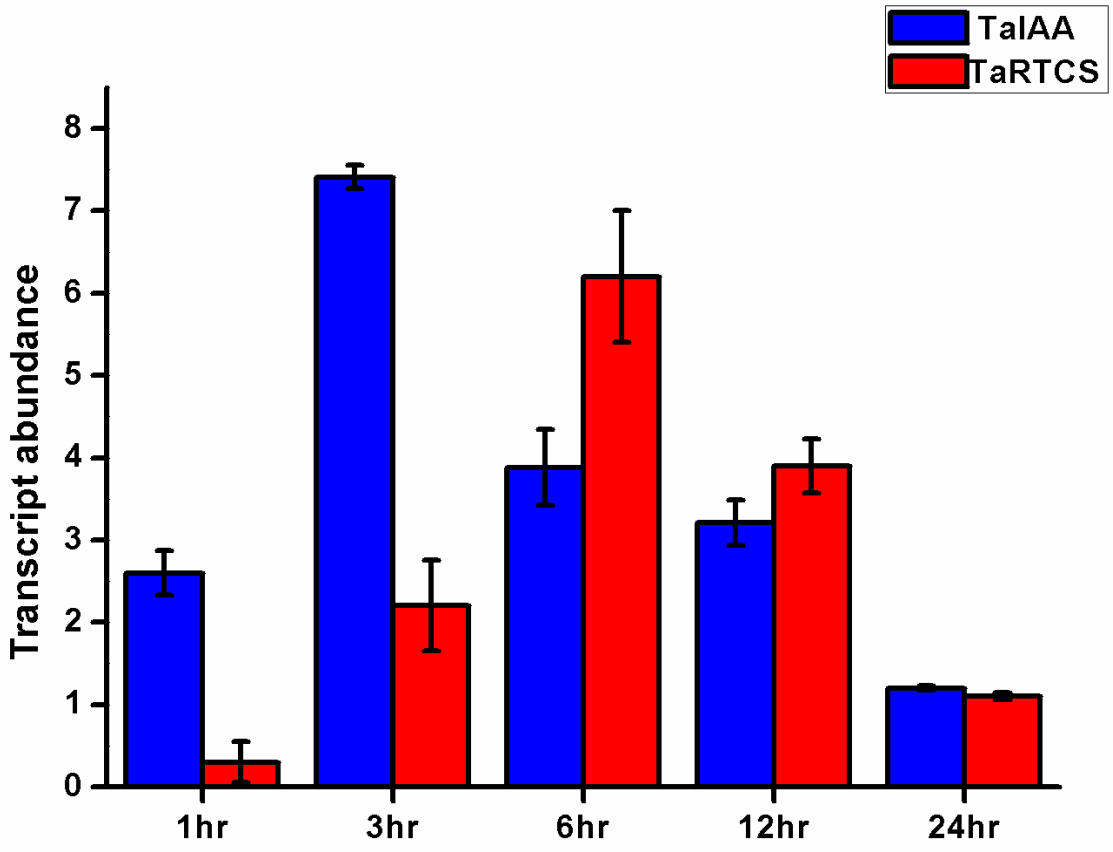

Fig. 4.28. Graphical representation of the expression of *TaIAA* and *TaRTCS* genes in 10-day-old seedlings of Chinese spring variety of wheat with respect to control on exposure to indole-3 acetic acid (IAA) at the indicated time. The error bars represent the standard deviations of the means (n=5).

Chapter 5

Discussion

5. DISCUSSION

Plant root architecture is the critical agronomic trait that determines the productivity and environmental stress adaptability in crops [44]. Limited information is available on the molecular genetics of root architecture development and few genes are known to be involved in root development. The present work on commercial varieties of wheat has demonstrated the occurrence of genetic variation for nitrate deficiency tolerance in this crop. This finding is in agreement with the results obtained by Fathi [68]. Wheat cultivars tolerant to nitrate stress of 0 mM were identified by Fathi in the year 2008 [68]. In the present study, changes in nitrate concentration showed contrasting effects on both primary root length and lateral root density of different wheat cultivars but no significant difference was observed in the shoots. These results were in agreement with that of Linkohr and coworkers where they found that changes in the level of nitrate and phosphate could affect the growth of primary root length and lateral roots in *Arabidopsis* [135].

Stress tolerance has been reported to be positively correlated with the activities of antioxidant enzymes, namely, catalase, ascorbate peroxidase, superoxide dismutase and glutathione peroxidase in several plant species [6, 155, 156]. In the present work, the specific activities of SOD and POD enzymes were found increased in the nitrate tolerant cultivar WH 1021 under nitrate stress. These enzymes appear to play a role in nitrate deficiency tolerance of wheat by detoxifying the reactive oxygen species produced during nitrate stress.

MnSOD enzyme is one of the key enzymes of defense mechanism which, is activated during environmental stress in wheat [11]. In the present study, the changes in the level of expression of *MnSOD* gene in the roots of wheat cultivars PBW343 and WH1021 in response to nitrate stress were observed. RT-PCR results revealed that the expression of *MnSOD* gene was significantly higher in WH1021 cultivar (3 folds) as compared to the PBW343 cultivar (1.58 folds) with respect to control in each nitrate stress (Fig. 4.6-c). The up-regulation of SOD gene under different stresses has been reported by previous workers in *Arabidopsis* [230], rice [19] and *Sorghum* [199]. It seems that roots sensed the depletion of nitrate, thus signaling molecular mechanism to activate the genes regulating the antioxidant enzyme production in order to protect plant against the oxidative damages produced due to nitrate stress.

NRT2.1 is a high affinity transporter gene which is being activated at low nitrates level to the plant [119]. An increased expression of *TaNRT2* gene coding for *NRT2.1* transporter was found in the nitrate tolerant wheat plants under nitrate concentration of 0.5 mM. It appears that a little amount or no nitrate could activate the expression of *NRT2* gene in wheat at low nitrate atmosphere. Similar observations were obtained in contrasting rice varieties by Araujo *et al* [7].

GLU3 gene was considered to be involved in cellulose synthesis in roots and shoots in grass family [27], structural changes in cell wall in the internodes of rice [257] and regulates seed germination, dormancy and in the defense against seed pathogens [131]. The cellulose content in roots can be altered in response to phosphate and nitrate stress due to carbon partitioning in rice [252]. In the present study, the differential gene expression of *TaGLU3* was two folds higher in PBW343 cultivar than WH1021 cultivar. Based on the results obtained, it seems that nitrate starvation could induce carbon partitioning in the roots, favoring them to elongate because of the induction of cellulose synthesis.

The expansin gene modulate cell walls in dicots by increasing the cellulose and xyloglucan content [213] whereas in monocots they might be involved in lignification via the process of ferulic acid cross linking [144]. An increase in the expression of *TaEXP* gene expression under nitrate stress in the sensitive cultivar was observed. Also the root length in PBW343 was significantly higher at 0mM nitrate concentration. Similar findings were observed by Guo and coworkers in soybean where the changes in the expression of *GmEXPB2* were recorded due to the alterations in root architecture under abiotic stress [81].

The *RTCS* gene and its orthologs are responsible for post embryonic root development in plants. The isolation and characterization of *RTCS* gene and its ortholog have been reported in maize [90], rice [99] and *Arabidopsis* [204]. In these reports, genetic and functional characterization was done on the base of recessive loss-of-function mutation. The present study showed the *in-silico* identification of orthologs of maize *RTCS* gene in eleven plant species and the functional characterization of the ortholog (*TaRTCS*) of this gene in wheat by reverse genetics.

The *in-silico* comparative study of the maize orthologs of *RTCS* gene in 5 monocots and 6 dicots revealed that each of these orthologs had two exons separated by a single intron. From this analysis, it was found that *TaRTCS* gene structure was similar to the gene structure of maize *RTCS* gene [214] and rice *CRL1* gene. On the basis of the presence of two exons in both monocots and dicots, the mechanism for the post-transcriptional expression of *RTCS* gene in all the studied plants species appears to be the same.

The true orthologs of ZmRTCS from the 5 monocots had shown high amino acid (aa) sequence query coverage and identity. Further, the evaluation of RTCS protein sequence of 6 dicots showed that there was high aa conservation in the first half of the protein but variability was higher in the second half. It seems that during the course of evolution, first exon of *RTCS* gene was conserved in all the orthologs.

These results showed that the RTCS protein carried an LOB domain which was further subdivided into 3 functional motifs i.e C-motif, GAS-motif and leucine zipper-like motif. The consensus sequence $Cx_{2-3}Cx_nCx_{2-3}C$ (n >12) in the C-motif of LOB domain of RTCS protein was found conserved in all the studied species (Fig 4.9.). Alanine was present at the position 13 of C-motif in dicots except *S. tuberosum*. Valine was present at this position in monocots, however, valine was replaced by alanine in TaRTCS. Iwakawa and coworkers observed that the variation in C-motif in *Arabidopsis* was one of the factors in determining the function of *AS2* gene (an ortholog of maize *RTCS*) [104]. It appears that C-motif may be playing an important role in the function of *RTCS* gene in wheat.

The hydrophobic leucine-zipper motif was conserved in all the studied plant species (Fig. 4.10.). The leucine-zipper is involved in protein–protein interactions in yeast [63] and plants [145]. The hydrophobic residues in the leucine-zipper motif may be providing thermal stability during protein-protein interactions [103]. Hence, the hydrophobic leucine-zipper motif may be playing an important role in the function of RTCS protein.

The GC content of a gene is one of the important criteria to determine the evolutionary bases of a particular gene. The present study revealed a significant difference in the GC content of monocots and dicots. A bimodal distribution of GC was found in monocots whereas the dicots

had a unimodal distribution. The results are in accordance with the previous findings of genes studied in different plant species [28, 225]. The possible reason of this bimodality in *RTCS* gene among monocots could be due to either positive Darwinian selection, acting at the level of translational efficiency or neutral mutational bias [215].

The value of π (average number of nucleotide differences per site between two sequences) and θ (number of segregating polymorphic sites) obtained by statistical analysis of *ZmRTCS* gene and its ortholog sequences, was found to be 0.70015 and 0.90403, respectively (Table 4.8.). Since the values of π and θ were not similar, thus it can be concluded that there was a genetic drift mutation disbalance among the species. As per previous studies, these two estimates are expected to give similar values under a drift– mutation balance; otherwise, any form of natural selection may explain the maintenance of genetic variation [85].

Wolfe and coworkers have reported that the monocots have diverged around 200 million years ago (Myr) from dicots [237]. The aa sequence analysis of RTCS protein in the studied species revealed the presence of two clades, dicots and monocots. On the basis of protein similarity, monocots clade was further divided into three subgroups; first (ZmRTCS and SbRTCS), second (SiRTCS and PvRTCS) and third (TaRTCS and OsRTCS) (Fig.4.14.). The first subgroup showed more divergence to subgroup three as compared to subgroup two. These findings are in agreement with the evolutionary history of these plant species [23, 32, 223]. The recently diverged species were found to be closely related.

In this study on wheat plants, the *RTCS* gene showed preferentially more expression in seedling roots, germinating roots and crown as compared to the leaves. The analysis of EST database of wheat plants as described in results under section 4.5 also supported this conclusion. The expression of *RTCS* gene has been reported in the roots of maize and rice [90, 137].

The maize *RTCS* gene was located on chromosome 1 [90] and rice *CRL1* gene was located on chromosome 3 [99]. In the present study, wheat RTCS gene was located on 4A, 4B and 4D chromosomes (Fig.4.16.) indicating the presence of at least 3 copies of this gene in wheat. Due to the unavailability of complete genome sequences of wheat, it is not possible to determine which copy (4A/4B/4D) matches closely with Zm*RTCS* gene. The results obtained

from physical mapping of *RTCS* gene and its ortholog in rice and wheat are in line with the model proposed by Devos and coworkers that indicates a close synteny between the chromosome 1 of maize, 3 of rice and 4 of wheat [53]. Bai *et al*. found a major QTL for root length on wheat chromosome arm 4D in their study using QTL analysis and found that this QTL was located between RhtMrkD1.4D—wPt-0431.4B4D/wPt- 5809.4B4D [12] . This QTL was associated with increase in total root length, root surface area, root dry weight, root/shoot ratio and root volume under normal conditions. Since a copy of *TaRTCS* gene was found on chromosome 4D, there is a possibility of this gene to lie in between the locus RhtMrkD1.4D and wPt-0431.4B4D/wPt-5809. To confirm the exact location and to use *TaRTCS* in future breeding programs, fine mapping studies can be carried out using sequence resources identified in the present study.

In the present study, the functional characterization of the Ta*RTCS* gene was done by virus induced gene silencing approach. A BSMV-based antisense construct having a unique region of *TaRTCS* gene effectively targeted the root RTCS gene and lead to a decrease in the root length, lateral roots and root surface area (Fig 4.27.). A significant reduction in the lateral roots was also observed in silenced plants as compared to the control plants. Similar type of phenotypic changes were reported in RTCS mutant of maize[214] and CRL1 mutant of rice [101]. Hence, TaRTCS appears to be involved in determining root architecture in wheat. Further, the real time analysis showed 66 % reduction in TaRTCS transcript and 82 % reduction in PDS transcript in silenced plants as compared to the control plants (Table 4.10.). Similar observations were recorded when *TaCOI1* gene, which is also a root specific gene, was silenced in wheat [15]. Thus, the present study provides an evidence that genes expressing in roots can be effectively studied by the BSMV-based VIGS approach. Major drawbacks of the VIGS approach is that it induces disease symptoms associated with virus and masks the exact phenotype of the silenced gene under study. Hence, other approach such as *Agrobacterium* mediated transformation should be employed to determine the exact function of *RTCS* gene in wheat.

Almost equal expression of *TaRTCS* and *TaIAA* genes on exposure to indole acetic acid (auxin) (Fig.4.28.) indicated that the RTCS gene belongs to the family of genes that are activated by auxin responsive factors in its promoter region. The CRL1 gene in rice [101] and LBD16 gene in *Arabidopsis* promoter regions [174] have also been reported to be activated by auxin response

factor, suggesting a conserved regulatory mechanism. Ariel *et al* observed high expression of *LBD1* gene in *Medicago* under environmental stress [8]. The LBD1 gene targets LOB domain which is also present in *RTCS* (Fig.4.9.), indicating that *RTCS* gene in wheat may also be regulated under abiotic stress. On the basis of results obtained, it can be hypothesized that stress (biotic or abiotic) leads to the activation of some signals that prevents the auxins to accumulate at lateral root initiation zone in plants. Since auxins are involved in the expression of *RTCS* gene as shown above, therefore if the above hypothesis stands true, this may be a further step to determine the genes involved in upstream and downstream region of the wheat *RTCS* gene.

This study is the first report on the identification and characterization of the *RTCS* gene in wheat.The comparative study of *RTCS* gene among different plant species in the study provide a bioinformatics approach for studying the genes related to root architecture. Also, the genes involved in the root architecture under nitrate stress studied in the present work gives a basic idea about the pathways involved. These results open up new opportunities for the comparative genomics, identification of genes and pathways involved for the root architecture modification in wheat under stress.

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6. REFERENCES

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APPENDIX

Nucleotide sequences of the maize RTCS and its orthologs used in the study

>ZmRTCS

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