Chapter 6

Comparative structural analysis of various functional domains of Histidine Kinase (HK) and phosphotransfer proteins (HPt) in Oryza sativa and Arabidopsis thaliana.

6.1 Background

Throughout the life cycle, organisms continuously respond to environmental signals which lead to the change in their physiology, morphology and development. The sensory and transduction systems evolved in bacteria enable them to survive and adapt to various environmental conditions. Two principle components plays a major role in the signal transduction pathway of the plant cell, Ca^{2+} and protein kinases (enzyme that phosphorylates and thereby alters the activity of target protein) although many signals interact cooperatively and synergistically with each other to produce complex plant responses. It is believed that the modular signaling components are interwined within several signaling pathways (Stock et al. 2000). In eukaryotes, His-Asp phosphorelays are coupled to
6.1 Background

MAP kinase cascades (Chang et al. 1993; Maeda et al. 1994; Shieh et al. 1997) and cAMP-dependent protein kinase (Shaulsky et al. 1996; Shaulsky et al. 1998; Thomason et al. 1998).

The existence of evolved surface-exposed signal transduction system, which comprise of transmembrane (TM) proteins that channel the input from sensory modules to intracellular responses has been established (Mascher et al. 2006). These TM signaling system includes the chemotaxis receptors, anti-σ:σ factor pairs, Ser/Thr protein kinases, and histidine protein kinases together with their cognate response regulators which are well known as “Two Component System (TCS)” (Mascher et al. 2006). Majority of eubacterial systems has His-Asp phosphotransfer systems but were rare in eukaryotes, in which kinase cascade involving Ser/Thr and Tyr phosphorylation predominates (Stock et al. 2000).

The functional state of the basic two-component phosphoryl transfer signal transduction pathway involves three phosphotransfer reactions and two phosphoprotein intermediates:

- the autophosphorylation of a conserved histidine in the transmitter domain of the sensor,

- the phosphotransfer to a conserved aspartate in the receiver domain of the RR (by the activity of RR), and

- the dephosphorylation of the RR to set the system back to the prestimulus state (Parkinson 1993; Stock et al. 1995)

All the bacterial genome sequenced so far, with the exception of Mycoplasma species, were found to have gene encoding TCS proteins (Mascher et al. 2006). Genome wide analysis have identified two component system members in various bacterial species such as B. subtilis (Fabret et al. 1999), E. coli (Mizuno 1997), P. aeruginosa (Roddig et al. 2000), C. glutamicum (Kocan et al. 2006), S. coelicolor (Hutchings et al. 2004), and Cyanobacteria (Ashby & Houmard 2006).

The prototypical two component system consist of a Histidine Kinase (HK) protein, containing a conserved kinase core, and a Response Regulator (RR)
6.1 Background

Figure 6.1: Schematic representation of TCS system. (a) Simple TCS member. Sensing of an extracellular signal is initiated by the input domain of sensory HK, phosphorylating the conserved His in its transmitter domain (TD). In the next step, the conserved Asp in the receiver domain of the Response Regulator (RR) is autophosphorylated, resulting in the signal output. (b) A hybrid-type TCS which the conserved His and Asp are found in the same protein, which serves as the sensory HK and is usually membrane bound. The non membrane bound Hpt acts as a mediator for the transfer of the phosphoryl group between the HK and the RR.

protein, containing a conserved regulatory domain. The HK harbors an N-terminal input domain that senses a specific stimulus by binding to a signaling molecule. The information is then transduced through intramolecular conformational changes resulting in activation of cytoplasmic transmitter domain (Wolanin et al. 2002). The transmitter, in turn, activates the cognate receiver,
encoded by the N-terminal domain of the RR.

Histidine kinase protein is a dimeric protein and is present in all the three major kingdoms of life (the bacteria, Archea and Eukarya) (Grebe & Stock 1999; Koretke et al. 2000). In Eukarya, HK appear to be confined to plants and to free-living organisms such as yeast, fungi and protozoa (Thomason & Kay 2000). Till date various HK proteins have been sequenced in various organisms such as EnvZ (Mizuno et al. 1982), PhoR (Tommassen et al. 1982), CpxA (Albin et al. 1986), NtrB (Nixon et al. 1986), DctB (Rouson et al. 1987), VirA (Leroux et al. 1987), and CheA (Stock et al. 1988). HKs have been cloned in plants such as Arabidopsis (Challg et al. 1993; Huct et al. 1995; Kakimoto 1996; Hua. et al. 1998), tomato (Payton et al. 1996; Lashbrook et al. 1998), and R. palustris (Vriezen et al. 1997). Genome-wide analysis have characterized TCS gene family members in Arabidopsis (Hwang & Sheen 2001; Grefen & Harter 2004) and rice (Pareek et al. 2006). In Arabidopsis, TCS mediates the photosensitivity (Schneider-poetsch et al. 1991; Yeh & Lagarias 1998), ethylene response (Hwang & Sheen 2001) and also acts as a putative osmosensor (Urao et al. 1999). Soon after the discovery of sensor HK, members of RR have been characterized in Arabidopsis and Zea mays (Sakakibara et al. 1998; Sakakibara et al. 1999).

HKs can be subdivided into two broad classes according to the organization of the conserved sequence boxes into separate domains (Grebe & Stock 1999). Class I HKs have the H-box containing domain adjacent to the ATP binding domain. EnvZ, an osmosensor, is the representative example of this class. Class II HKs have H-box containing domain separated from the ATP binding domain by some other domains, like in CheA, chemotactic sensor protein H-box containing P1 is separated from the ATP binding P4 by an intermediary P2 domain (Bilwes et al. 2003). The structures of the H-box domain of the osmosensor, EnvZ (Tomomori et al. 1999) and the P1 domain of CheA (Zhou & Dalquilst 1997) have been determined.

The RR regulatory domain was found to be a conserved ~120 residue domain (Volz 1993). Among the signaling proteins RRs holds a unique place in using Asp phosphorylation for regulation. The RR gives rise to the appropriate cellular response, which is mediated by the C-terminal effector (or output) domain of the RR through protein-protein interaction or protein-DNA interaction leading
to differential gene expression (Mascher et al. 2006). Analysis of various RRs in *Arabidopsis* showed that they can be classified into two distinct subtypes, type-A and type-B regulators, based on their structure, biochemical properties, and expression profiles (Imamura et al. 1999).

Histidine kinase activity is regulated by receptor-ligand interactions. Analysis of various phosphorelay system shows that the mechanism are of two types. The simpler version which were found to be prevalent in bacteria and is often called the two component system. Here, a histidine kinase and a response regulator directly relay phosphate to one another. The other class uses an expanded multistep His-Asp-His-Asp relay mechanism (Appleby et al. 1996). They most commonly employ a histidine kinase that has an attached receiver domain: this is known as a hybrid kinase (Figure 6.1). Analysis of various RRs shows that regulatory domains are not always attached to effector domains. Some exist as separate proteins at the ends of pathways where they mediate intermolecular regulation of output responses, while others are used in phosphorelay pathways as intermediates or as domains of hybrid HKs. Structural analysis of the bacterial chemotaxis protein CheY, an RR that lacks an effector domain, defined the fold of the regulatory domain (Stock et al. 1989).

In hybrid type kinases, phosphate is first transferred from the histidine residue in the transmitter to the aspartate residue of the attached receiver domain, then to a histidine residue on a histidine phosphotransfer domain (Figure 6.1). Finally, the phosphate is relayed from the HPt domain to the receiver domain of a downstream response regulator protein, which results in the output response. Analysis of all HKs shows that the protein posses conserved sequence fingerprints, termed as H, N, D, F and G-boxes, based on the highly conserved residue. It was observed that H-box bears the histidine that get phosphorylated while the N, D, F and G-boxes are located at the ATP binding site (Kofoid & Parkinson 1988; Stock et al. 1988; Stock et al. 1995).

Various studies performed on the HKs found in eukaryotes and prokaryotes shows noticeable differences such as hybrid HKs that contain RR domains are rare in prokaryotes (5 of 30 in *E. coli*) (Mizuno 1997), whereas eukaryotic HKs are almost exclusively hybrid kinases; the only known exception is *Arabidopsis* ERS (Yen et al. 1995).
Phosphotransfer (HPt) proteins which is the third component in His-Asp phosphorelays, have been identified in plants: AHP1 to AHP3 (ATHP1 to ATHP3) in Arabidopsis (Miyata et al. 1998; Suzuki et al. 1998; Imamura et al. 1999).

Analysis of hybrid TCS system in Arabidopsis showed interaction of ATHK1 with AHPT1 protein (Urao et al. 1999) and in rice OsHK3b with OsHPt4 protein (Unpublished data). With the availability of sequences of various HKs and HPts, the structure of various functional domains of HK proteins in plants especially Oryza sativa and Arabidopsis thaliana can give an insight to the molecular mechanism of signal transmission across the membrane from the sensing domain to the kinase core. Structure of phosphotransfer proteins (HPt) and its interaction with RR domain can further help us in understanding the mechanism of their functionality in a cell.

6.2 In silico modeling and analysis of various functional domains of HK and HPt proteins in Oryza sativa and Arabidopsis thaliana

6.2.1 Methodology

The sequences of OsHK3b (LOC.Os01g69920.2) and OsHPt4 (LOC.Os05g9410) from TIGR rice database (ver 6.1) and sequences of AtHK1 (At2g17820.1) and AtHPt1 (At3g29350) in Arabidopsis (TAIR ver 8.0) were used for modeling and analysis of various functional domains and their interactions.

Figure 6.2: Domain structure of hybrid type sensory HK protein in Arabidopsis thaliana (AtHK1) and Oryza sativa (OsHK3b).
The conserved domains of the sequences were identified from Pfam (Protein family database (Finn et al. 2006)). The results obtained from Pfam showed the presence of major signatures domain in the HK sequences (Figure 6.2). The first signature domain is the CHASE signaling domain, followed by transmembrane (TM) domain. Transmitter domain in HK was observed to act as a ATP binding domain. The conserved domain searched against the conserved domain database, CDD (Marchler-Bauer et al. 2009), also supported the Pfam results. The secondary structures of the domains and the protein were predicted using JNET secondary structure prediction server (http://barton.ebi.ac.uk/jpred2.html) (Cuff & Barton 1999; Cole et al. 2008), PREDATOR (Protein secondary structure prediction from sequences) (Kabsch & Sander 1983; Frishman & Argos 1995; Frishman & Argos 1996; Frishman & Argos 1997), STRIDE (STRuctural IDEntification) (Frishman & Argos 1995), PSIPRED (Protein Structure Prediction server) (http://bioinf.cs.ucl.ac.uk) (Bryson et al. 2005). The fold recognition analysis were performed using FUGUE (Sequence structure homology recognition) (Shi et al. 2001), GenTHREADER (Jones 1999; McGuffin & Jones 2003), 3DPSSM (Kelley et al. 2000) and phyre server (http://www.sbg.bio.ic.ac.uk). The architectural motifs and the topology of proteins with known 3D structure were analysed according to SCOP (Structure Classification Of Protein) (Murzin et al. 1995) and CATH (Orengo et al. 1997; Cuff et al. 2009) classifications. The three-dimensional structure of all the domains of HK and HPt proteins in rice and Arabidopsis were modeled in a stepwise procedure, starting with the identification of template structures. The identified templates were obtained from PDB and were aligned using structure alignment software STAMP (Russell & Barton 1992). This alignment was then used as a profile for aligning the target sequence using ClustalX (Larkin et al. 2007) for modeling various domains. The alignment was subjected to manual adjustments for the conserved structural motifs. For modeling various domains of HK and HPt protein, automated comparative protein modeling program Modeller9v3 (Sali & Blundell 1993; Eswar et al. 2000; Fisher et al. 2000; Marti-Renom et al. 2000) was used to generate a 100 all-atom model. The best model was chosen on the basis of the stereochemistry quality report generated using PROCHECK (Morris et al. 1992; Laskowski et al. 1993) and side chains of the modeled protein were optimized.
using SCWRL 3.0 (Canutescu et al. 2003). G-factor score was considered as a parameter for obtaining the quality of the modeled domain, and was obtained using PROCHECK. G-factor is essentially a log odds score based on the observed distribution of stereochemical parameters such as main chain bond angles, bond length and phi-psi torsion angles. The score for G-factors should be above -0.50 for a reliable model. The bond distances and dihedral angle restraints on the target sequences were derived from its alignment with the template three-dimensional structures. The spatial restraints and the energy minimization steps were performed within Modeller using the CHARMM22 force field for proper stereochemistry of proteins. Since the domain sequence used for the alignment and modeling have well conserved structural motifs and regions, and functional information is also available, the problem of low-sequence identity could be overcome, i.e. a multiple sequence alignment obtained from the known sequences can provide a reasonable approach to comparative structure modeling. Earlier attempts have been made to model the protein sequence having low identity with the template sequence (Singh et al. 2008). In order to verify the quality of the sequence alignment and optimise the position of gaps, corresponding positions from secondary structures were used. Further evaluation of the modeled domain and protein structures was done using the PROSA-web (Sippl 1993; Weiderstaein & Sippi 2007). Ramachandran plots were generated for both domain and protein structures to determine deviations from normal bond lengths, dihedrals and nonbonded atom-atom distances. Molecular visualization and analysis of the final model were carried out with Visual Molecular Dynamics (VMD) (Humphrey et al. 1996).

6.3 Result and Discussion

6.3.1 Modeling and analysis of CHASE domain of AtHK1 and OsHK3b protein

Various analysis have shown that the signal ligand binding to sensor domains of HKs normally results in the induction of an ATP dependent autophosphorylation of the HK domain in the catalytic core of the enzyme. Many cy-
toplasmic elements of the sensor complex have been reported earlier such as PAS (PER, ARNT, SIM), GAF (c-GMP-specific and c-GMP-stimulated phosphodiesterases, Anabaena adenylate cyclases and E. coli FhlA), and HAMP (for histidine kinases, adenylyl cyclases, methylaccepting proteins, and other prokaryotic signaling proteins) domains (Galperin et al. 2001). Several attempts have been made to analyze the structures of PAS and GAF domains. Recently the solution structure of a HAMP domain containing protein Af1503 from Archaeoglobus fulgidus has now been solved (Hulko et al. 2006). In recent years, several periplasmic signaling, HK ligand-binding structures have been solved such as PhoQ in Salmonella typhimurium (Cho et al. 2006), LuxQ, the quorum sensor of Vibrio harveyi (Neiditch et al. 2005; Neiditch et al. 2006), and citrate and fumarate sensors of E. coli and Klebsiella pneumonia CitA and DcusS, respectively (Reinelt et al. 2003; Pappalardo et al. 2003).

Analysis of N-terminal region of HK protein revealed the presence of CHASE (Cyclases/Histidine kinases Associated Sensory Extracellular) signaling domain. These domains were reported to be found in diverse receptor and like proteins with histidine kinase and nucleotide cyclase domains. CHASE domain is an extracellular domain of 200-230 amino acids, which was found in transmembrane receptors from bacteria, lower eukaryotes and plants in the N-terminal region of HKs, adenylate cyclases, and predicted diguanylate cyclases/phosphodiesterases. Based on sequence conservation various types of CHASE (CHASE1 to CHASE6) domain have been reported. CHASE2 domains occur in serine/threonine kinases, and CHASE3 domains occur in Methyl accepting Chemotaxis Proteins (MCP). Till date, only one bacterial CHASE domain-containing HK has been analyzed with respect to its biological function: VsrA, a CHASE3 domain-containing HK, is required for the expression of virulence factors in Pseudomonas solanacearum (Schell et al. 1994). Analysis of the CyaA adenylate cyclase from Myxococcus xanthus indicates that CHASE2 domain might function in osmosensing mechanism (ZhuLin et al. 2003). CHASE domains were reported to bind with diverse low molecular weight ligands, such as cytokinin-like adenine derivatives or peptides, and mediate signal transduction through the respective receptors (Pas et al. 2004). Although the CHASE domain was characterized by the mechanism of ligand binding, evolutionary relationships between other sensing domains
remains unrevealed. With the view to understand characteristics of the CHASE domain, sequence and structural analysis of the domain has been performed.

Sequence analysis of CHASE domains in the sensory HK protein of *A. thaliana* and rice revealed the presence of conserved residues. Using BLAST and 3DPSSM, distant homologs were identified for CHASE domains sequence of AtHK1 and OsHK3b proteins. Analysis of various signaling domains, it was hypothesized earlier that sensing domains may have diverged during evolution but still possess the same folds (Anantharaman & Aravind 2001; Aravind *et al.* 2002).

The template structures (1H4P.pdb, 1KKT.pdb, 1RZ3.pdb and 2Z1Q.pdb) for modeling AtHK1 and OsHK3b CHASE domain were obtained from PDB. The template structures were observed to possess sensor kinase like secondary structure folds. The CHASE domains of AtHK1 protein in *A. thaliana* and OsHK3b in rice were modeled using comparative modeling approach (see Method). The Ramachandran plot analysis showed that the modeled OsHK3b CHASE domain has 95.9% residues in most favourable regions with the remaining 2.5% of residues occurring in generously allowed regions while only 1.6% residues were found in disallowed region. While in AtHK1, CHASE domain structure, 95.2% residues were found in most favourable regions with the remaining 1.9% of residues occurring in generously allowed regions while 2.9% residues were found in disallowed region (Figure 6.3(a) and 6.3(b)). The PROCHECK result summary showed 10 out of 117 residues labeled in CHASE domain structure of AtHK1 while in CHASE domain of OsHK3b, 9 out of 138 residues were found to be labeled. The torsion angles of the side chain designated by $\chi_1-\chi_2$ plots showed only 1 labeled residues out of 67 in AtHK1 CHASE domain while in OsHK3b CHASE domain, 1 out of 85 was found labeled.

All main-chain and side-chain parameters were found to be in the ‘better’ region. The G-factor scores of the OsHK3b CHASE domain model was observed to be -0.13 for dihedral bonds, -0.49 for covalent bonds and -0.43 overall while in AtHK1 CHASE domain, G-score was found to be -0.15 for dihedral bonds, -0.41 for covalent bonds and -0.38 overall. The distribution of the main chain bond lengths and bond angles were 99.1% and 98.8% within limits for the modeled AtHK1 CHASE domain while for OsHK3b CHASE domain, main chain bond lengths and bond angles were found to be 98.8% and 90.7% within the limit.
6.3 Result and Discussion

Figure 6.3: Ramachandran plot of $\psi/\phi$ distribution of CHASE domain residues in modeled AtHK1 (a) and OsHK3b (b) protein produced by PROCHECK.

Conclusively, the modeled structure of HK CHASE domain in both rice and Arabidopsis was found comparable to the structurally resolved template structure as structural motifs were found conserved. The PROSA-web energy plots for CHASE domains of AtHK1 and OsHK3b protein showed z-score for pair, surface and combined energy which was found to be -2.57 and -3.54 respectively (Figure 6.4).

The secondary structure of OsHK3b CHASE domain consists of six $\beta$-sheets and four $\alpha$-helix structure while AtHK1 CHASE domain secondary structure was observed to consist of six $\beta$-sheets and two $\alpha$-helix structures. Due to low similarity of the CHASE domain sequence with template and other known signaling molecules, secondary structures were not predicted for the N-terminal region of the CHASE domain for both AtHK1 and OsHK3b. The CHASE domain of both AtHK1 and OsHK3b has been predicted to have $\alpha+\beta$ fold, with two extended helices on both boundaries and two central helices separated by sheets (Figure 6.5). The termini of the CHASE domain in both AtHK1 and OsHK3b were found less conserved as compared with the central part of the domain.
6.3 Result and Discussion

(a) Energy plot of the modeled CHASE domain of AtHK1 protein
(b) z-score plot of the modeled CHASE domain of AtHK1 protein

(c) Energy plot of the modeled CHASE domain of OsHK3b protein
(d) z-score plot of the modeled CHASE domain of OsHK3b protein

Figure 6.4: PROSA-web analysis of modeled CHASE domain in AtHK1 and OsHK3b protein confirms the quality of the modeled structure.
6.3 Result and Discussion

6.3.2 Comparative modeling of transmitter domains (TD) of AtHK1 and OsHK3b protein

To create a model of transmitter domain of AtHK1 and OsHK3b proteins, BLAST searches were first performed against the PDB for proteins with similar sequence and known 3D structures using the 280 amino acid and 288 amino acid sequence of TD. The search identified structures of 2C2A.pdb from *Thermotoga maritima* (ADP bound); chain A, 3D36.pdb from *Geobacillus stearothermophilus* (ADP bound) can be considered as possible templates for modeling both the domains using the threading approach (see Method). The ramachandran plot for the modeled TD in OsHK3b showed 95.1% residues in most favourable regions with the remaining 4.5% of residues occurring in generously allowed regions while only 0.4% residues were found in disallowed region. While for modeled TD of AtHK1, 95.4% residues were found in most favourable regions with the remaining 2.7% of residues occurring in generously allowed regions while 1.9% residues were found
6.3 Result and Discussion

Figure 6.6: Ramachandran plot of $\psi/\phi$ distribution of modeled transmitter domain residues of AtHK1 (a), and OsHK3b (b) produced by PROCHECK.

in disallowed region (Figure 6.6). The PROCHECK result summary showed 10 out of 288 residues labeled in TD of AtHK1 while in OsHK3b TD, 8 out of 276 residues were found to be labeled. The torsion angles of the side chain designated by $\chi_1-\chi_2$ plots showed only 7 labeled residues out of 177 in AtHK1 TD while in OsHK3b TD 10 out of 176 were found labeled. All main-chain and side-chain parameters were found to be in the 'better' region.

The observed G-factor scores of the modeled TD in OsHK3b was found as -0.09 for dihedral bonds, -0.38 for covalent bonds and -0.2 overall while for the modeled TD of AtHK1, G-score was found to be -0.22 for dihedral bonds, -0.34 for covalent bonds and -0.42 overall. The distribution of the main chain bond lengths and bond angles were 98.7% and 97.5% within limits for the modeled TD of AtHK1 while for modeled OsHK3b TD main chain bond lengths and bond angles were found to be 98.0% and 96.7% within the limit. Conclusively, the modeled structure of TD in both OsHK3b and AtHK1 was found comparable to the structurally resolved template structure as structural motifs were found conserved. The PROSA-web energy plots for TD structures of AtHK1 and OsHK3b protein showed a z-score for pair, surface and combined energy which was found
to be -6.64 and -5.81 respectively (Figure 6.7).

### 6.3.3 Analysis of transmitter domain (TD) structure of AtHK1 and OsHK3b protein

Earlier some catalytic ATP binding domain structures have been determined by X-ray crystallography for the Thermotoga maritima (CheA), E. coli (PhoQ, NtrB) (Bilwes et al. 2003; Marina et al. 2001; Song et al. 2004). The EnvZ TD structure was found to have a mixed $\alpha/\beta$ sandwich fold made from five beta-strands and three $\alpha$-helices (Tomomori et al. 1999). Analysis of TD of AtHK1 showed presence of seven $\beta$-sheets namely $\beta_1$(73-76), $\beta_2$(100-103), $\beta_3$(110-113), $\beta_4$(143-147), $\beta_5$(214-218), $\beta_6$(267-274), $\beta_7$(278-285) and seven $\alpha$-helix namely $\alpha_1$(3-29), $\alpha_2$(34-65), $\alpha_3$(77-92), $\alpha_4$(115-131), $\alpha_5$(172-180), $\alpha_6$(228-233), $\alpha_7$(253-265) in the modeled structure. While TD of OsHK3b showed the presence of five $\beta$-sheets $\beta_1$(98-100), $\beta_2$(142-143), $\beta_3$(206-209), $\beta_4$(257-263), $\beta_5$(267-273) and seven $\alpha$-helix namely $\alpha_1$(9-29), $\alpha_2$(34-65), $\alpha_3$(77-95), $\alpha_4$(120-133), $\alpha_5$(190-194), $\alpha_6$(217-221), $\alpha_7$(242-254). The $\beta_6$ and $\beta_7$ were observed to be longer than any other $\beta$-sheet present in the secondary structure of TD in AtHK1 (Figure 6.8). The N-terminal $\alpha$-helix was found to be shorter than the one found in Thermotoga maritima in TD of both AtHK1 and OsHK3b. The kinase domain observed in TD were found structurally related to the ATP-binding domains of the GHL ATPase family (GyraseB, Hsp90 and MutL) and hence these ATPase forms the GHKL superfamily (Dutta & Iinoye 2000). The cytoplasmic domain structure in Thermotoga maritima shows a long $\alpha$-helix at the N-terminal region with a kink due to presence of Proline residue in the structural motif (Marina et al. 2005). In TD of AtHK1 and OsHK3b, proline residue was found conserved in the secondary structure of the domain but kink was observed only in TD of AtHK1.

Analysis of ADP binding to the TD of AtHK1 and OsHK3b showed various hydrogen bond interaction with the active site residues (Table 6.1 and 6.2)(Figure 6.9). The conserved Asp209 of TD in AtHK1 and Asp229 of TD in OsHK3b was observed to form hydrogen bond interaction with the adenine ring of ADP. Further analysis also showed hydrogen bond interaction between conserved leucine and glycine residues of TD in both AtHK1 and OsHK3b. Analysis of sequence
6.3 Result and Discussion

Figure 6.7: PROSA-web analysis of modeled TD of AtHK1 and OsHK3b confirmed the quality of the modeled structure.
6.3 Result and Discussion

Figure 6.8: Cartoon representation of transmitter domain of AtHK1 (a) and OsHK3b (b) showing the presence of conserved secondary structures.

Table 6.1: Hydrogen bonds between ADP and modeled TD of AtHK1. Hydrogen bond distances between the residues were calculated using HBPLUS.

<table>
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<th>ADP</th>
<th>TD residues</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
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<td>O2A</td>
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</tr>
<tr>
<td>O1B</td>
<td>Gly250</td>
<td>3.02</td>
</tr>
<tr>
<td>O3</td>
<td>Ala240</td>
<td>2.77</td>
</tr>
<tr>
<td>N6</td>
<td>Asp220</td>
<td>3.06</td>
</tr>
<tr>
<td>O3B</td>
<td>Gln172</td>
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</tr>
<tr>
<td>O2B</td>
<td>Gln172</td>
<td>2.61</td>
</tr>
</tbody>
</table>

Analysis of binding in TD with ADP revealed that β5 and β6 contributes majorly to the interaction with ADP in AtHK1 while in OsHK3b, α4, α5 and β5 contributes to the interaction with ADP (Figure 6.2). The interaction of ADP
6.3 Result and Discussion

Figure 6.9: Interaction of ADP and Mg$^{2+}$ with the transmitter domain residues of AtHK1 (a) and OsHK3b (b).

<table>
<thead>
<tr>
<th>ADP</th>
<th>TD residues</th>
<th>Distance (Å)</th>
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<tr>
<td>O2B</td>
<td>Asn128</td>
<td>2.98</td>
</tr>
</tbody>
</table>

Table 6.2: Hydrogen bonds between ADP and modeled TD of OsHK3b. Hydrogen bond distances between the residues were calculated using HBPLUS.

with conserved Asn128 was observed in TD of both AtHK1 and OsHK3b. Analysis of EnvZ NMR structures revealed the presence of Hsp90 and DNA gyrase B like folds responsible for the ATP binding. Similar secondary structures and residues were observed in the TD of AtHK1 and OsHK3b. The Asn124 was found
conserved in TD of both AtHK1 and OsHK3b. The motif G1(DxGxG\Phi)(220-224 in AtHK1 and 209-213 in OsHK3b) and G2 (G\PhiG\Phi) (222-224 in AtHK1 and 241-243 in OsHK3b) was found conserved in the TD. These motifs were observed to be close to the ATP binding site, similar to the Hsp90 and EnvZ structure. Previously identified conserved N, G1, F and G2 boxes were found conserved in the cytoplasmic domain in TD of both AtHK1 and OsHK3b. Previous mutagenesis studies have established that the glycine-rich regions, G1 and G2, are essential for kinase activity (Yang & Inouye 1993). Analaysis of Hsp90-ADP complex have shown that the Asn(residue 347 in E.coli and 37 in Hsp90) binds to the ADP. Similarly, in TD of OsHK3b, conserved Asn128 was observed to form hydrogen bond interaction with the ADP while in TD of AtHK1 conserved Asn128 was observed to form an non bonded interaction with ADP. In an earlier study, the ATP dependent autokinase activity was observed to be lost when Asn347 was mutated to Asp in EnvZ mutant however similar mutation study in OmpR, autokinase activity was retained.

The vast number of crystal structures ATP binding domains of His-Kinase like proteins have aided in the identification of conserved and varied functional
features at their active sites subsequent to phosphorylation. Hence the modeled structures of TD of AtHK1 and OsHK3b protein will enhance the current understanding of the interaction of ADP with the conserved active site residues.

6.3.4 Modeling and analysis of RR domain of AtHK1 and OsHK3b

To create a model of RR domain of AtHK1 and OsHK3b protein, BLAST searches were made against PDB for proteins with similar sequence and known 3D structures using the 132 amino acid and 148 amino acid domain sequences respectively. The search resulted in identification of structures of RR in *Saccharomyces cerevisiae*, *Streptococcus pneumonia*, *Aspergillus oryzae* and *Thermotoga maritima* as possible templates for modeling RR domain of AtHK1 and OsHK3b protein. The structure of RR domain of AtHK1 and OsHK3b were predicted using comparative modeling strategy (see Methods).

![Ramachandran plot](image)

Figure 6.11: Ramachandran plot of $\psi/\phi$, distribution of RR domain residues in AtHK1 (a) and OsHK3b (b) model produced by PROCHECK.

The template protein structures used for the alignment and modeling were observed to have well conserved structural motifs and regions, thus the problem of
6.3 Result and Discussion

Low-sequence identity (40% to 55%) could be ignored i.e. multiple sequence alignment obtained for the known template structures provided a reasonable approach to comparative structure modeling of the RR domain in AtHK1 and OsHK3b. Ramachandran plot for the modeled structures showed that the RR domain of OsHK3b which is 132 residues long (Figure 6.11(a)) showed 95.6% residues in allowed region, 3.4% in generously allowed regions and only 0.9% in disallowed region while RR domain structure of RR domain in AtHK1, which is 148 residue long (Figure 6.11(b)) has 97.1% residues in allowed region, 2.2% in generously allowed region and only 0.7% in disallowed region. The PROCHECK result summary showed 8 out of 146 residues labeled in RR domain of AtHK1 while RR domain of OsHK3b, only 4 residues were found labeled out of 132 residues. The torsion angles of the side chain designated by $\chi_1$- $\chi_2$ plots showed only 2 labeled residues out of 87 in RR domain of AtHK1 while in RR domain structure of OsHK1, 2 out of 82 residues were found to be in labeled region. The observed G-factor scores of the present model were -0.07 for dihedral bonds, -0.35 for covalent bonds and -0.17 overall in RR domain structure in AtHK1 while in RR domain of OsHK3b, the observed G-factor scores of the model were -0.08 for dihedral bonds, -0.43 for covalent bonds and -0.20 overall.

The distribution of the main chain bond lengths and bond angles were 98.1% and 91.6% within limits for model of RR domain in AtHK1 and 98.8% and 90.1% within the limit for RR domain model of OsHK3b. This distribution in the bond angles is attributed to many residues that are not conserved in RR domain in comparison to the template structures. Conclusively, the modeled structure of both RR domains is comparable to the resolved RR domains structures, as structural motifs were found conserved. The PROSA-web energy plots for RR domains of AtHK1 and OsHK3b showed a z-score for pair, surface and combined energy was found to be -4.82 and -5.14 respectively (Figure 6.12). Regions of the secondary structure were also verified using PREDATOR and STRIDE software.
6.3 Result and Discussion

Figure 6.12: PROSA-web analysis of modeled RR domain of AtHK1 and OsHK3b confirmed the quality of the modeled structure.
6.3.5 Analysis of three dimensional model of RR domain in AtHK1 and OsHK3b

A comparison of the amino acid sequence of RR domains of both AtHK1 and OsHK3b with other experimentally resolved RR domains structures using various structural approaches revealed more than 30 conserved amino acid residues. Despite the very low level of sequence identity, RR domain predicted structures were observed to have retained the same overall fold. Analysis of RR domain of AtHK1 protein showed the existence of five $\beta$-sheets and six $\alpha$-helix (Table 6.4) (Figure 6.13(b)). While analysis of RR domain in OsHK3b showed the presence of five $\beta$-sheets and five $\alpha$-helices in its secondary structure (Table 6.3) (Figure 6.13(a)). The secondary structure of RR domain of AtHK1 showed an additional $\alpha$-helix with respect to the RR domain structure of OsHK3b protein.

<table>
<thead>
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<th>Secondary Structure</th>
<th>Amino Acid residue</th>
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<tbody>
<tr>
<td>$\beta_1$</td>
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</tr>
<tr>
<td>$\alpha_1$</td>
<td>8 to 21</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>26 to 28</td>
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</tr>
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</tr>
<tr>
<td>$\alpha_6$</td>
<td>140 to 147</td>
</tr>
</tbody>
</table>

Table 6.3: Secondary structures observed in RR domain of AtHK1 protein. The secondary structures are tabulated according to the order of appearance in the domain.

Earlier, analysis of crystal structure of RR domain from *Streptococcus pneumoniae* in both complex and native state has been performed (Bent *et al.* 2004). It was observed that the active site of RR domain showed the presence of con-
6.3 Result and Discussion

<table>
<thead>
<tr>
<th>Secondary Structure</th>
<th>Amino Acid residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1</td>
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</tr>
<tr>
<td>α1</td>
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<td>β5</td>
<td>117 to 120</td>
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<tr>
<td>α5</td>
<td>123 to 129</td>
</tr>
</tbody>
</table>

Table 6.4: Secondary structures in RR domain of OsHK3b protein. The secondary structures are tabulated according to the order of appearance in the domain.

erved Asp50 in OsHK3b and Asp82 in AtHK1 as compared to template Asp52 in Streptococcus pneumoniae. In OsHK3b RR domain, Asp57 conserved residue was found in α3 and β3 secondary structure motif while in AtHK1, Asp82 was found between β3 and α4 region. The conserved Lys136 in RR domain of AtHK1 and Lys121 in RR domain of OsHK3b has been predicted to play a major role in interaction with the phosphate group, like Lys109 in CheY, a bacterial chemotaxis protein. Analysis of structure of CheY RR domain showed that the fold is a doubly wound, five-stranded parallel sheet with topology β2-β1-β3-β4-β5 which was also observed in the RR domain structure of AtHK1 and OsHK3b.

Analysis of structure of RR domains like NtrC (Volkman et al. 1995), NarL (Baikalov et al. 1998), CheB (Djordjevic et al. 1998), FixJ (Gouet et al. 1999), ETR1 (Muller-Dieckmann et al. 1999), and Spo0A (Lewis et al. 1999) in other organisms have shown similar secondary structural features. It was observed that most unphosphorylated regulatory domains exist as monomers, but some dimerize (Robinson et al. 2000). Analysis of Arabidopsis ETR1 regulatory domain showed an additional β-strand at its C-terminus. This strand promotes dimerization by adding a sixth parallel strand to the β-sheet of its dimeric partner in an interaction involving α4, β5 and α5 of the second protomer (Muller-Dieckmann et al. 1999)
while the functional role of this dimerization is unknown (Robinson et al. 2000). It was postulated that phosphor-Asp bond might makes RR to undergo conformational change unlike P-Type ATPases. Analysis of experimentally determined structures revealed that the activated regulatory domains show conformational changes that primarily involve small repositioning of secondary structure elements that appear to be linked to the rearrangement of a specific set of side chains. Phosphorylation induces conformational changes that affect a large surface of the RR (Robinson et al. 2000). These structural changes were found responsible for the protein-protein interaction. It was observed that the specific sets of conserved residues were responsible for the transfer and the propagated conformational change leads to the protein-protein interaction. The analysis of RR domain in AtHK1 and OsHK3b protein revealed presence of similar conserved residues, hence it can be postulated that the mechanism of phosphorylation might be similar to the other known RR proteins.
6.3.6 Modeling and analysis of AtHPt1 and OsHPt4 protein

In order to model AtHPt1 and OsHPt4 proteins, BLAST searches were made against PDB for proteins with similar sequence and known 3D structures using the 104 amino acid and 133 amino acid domain sequences respectively. The search resulted in identification of structures of phosphotransfer protein in Zea mays and Oryza sativa as possible templates for modeling AtHPt1 and OsHPt4 proteins. Hence, the model of AtHPt1 and OsHPt4 protein was made using a comparative modeling strategy (see Method). The ramachandran plot analysis showed that the modeled OsHPt4 and AtHPt1 protein, which is 151 and 156 residues long respectively had 100% residues in allowed region (Figure 6.14). The PROCHECK result summary showed none of the residue as labeled both the modeled protein.

The torsion angles of the side chain designated by $\chi_1-\chi_2$ plots showed only 2 labeled residues out of 154 in AtHPt1 while in OsHPt4, 2 out of 110 residues were found to be in labeled region. The observed G-factor scores of the present model were 0.22 for dihedral bonds, -0.07 for covalent bonds and 0.11 overall in AtHPt1 protein structure while in OsHPt4, observed G-factor scores of the model were 0.21 for dihedral bonds, -0.10 for covalent bonds and 0.10 overall. The distribution of the main chain bond lengths and bond angles were 99.7% and 96.3% within limits for model of both AtHPt1 while for OsHPt4 protein structure the main chain bond lengths and bond angles were found to be 99.6% and 94.2% within the limit. Conclusively, the modeled structure of both AtHPt1 and OsHPt4 protein is comparable to the structurally resolved template structure as structural motifs are conserved. The PROSA-web energy plots for AtHPt1 and OsHPt4 protein shows a z-score for pair, surface and combined energy, was found to be -6.81 and -6.64 respectively (Figure 6.15).

A typical HPt domain was first discovered and analyzed in the E. coli ArcB sensor (Ishige et al. 1994; Tsuzuki et al. 1995), which is responsible for anaerobic responses (Iuchi et al. 1990; Iuchi 1993; Lynch & Lin 1996). Earlier well characterized eukaryotic two component system, Snlp-Ypdlp-Ssklp pathway, in which Ypdlp, comprising only a HPt domain, plays a crucial role (Posas et al. 1996). In
6.3 Result and Discussion

Figure 6.14: Ramachandran plot of $\psi/\phi$ distribution of residues in AtHPt1 (a) and OsHPt4 (b) model produced by PROCHECK.

recent years, the structures of four HPt domains/proteins have been solved. These are the P1 domain of *E. coli* CheA (Zhou *et al.* 1995b), HPt domain of *E. coli* ArcB (Kato *et al.* 1997), *B. subtilis* Spo0B protein (Varughese *et al.* 1998), and *S. cerevisiae* YPD1 protein (Xu & West 1999; Song *et al.* 1998). X-ray structure of ArcB revealed the presence of six $\alpha$-helices (A to F) with four helices forms a bundle in which conserved His717 was found located at helix-D. Analysis of HPts shows that they share a common four-helix bundle motif despite their overall lack of sequence similarity. The Hpt domain was observed to be homologous to the dimerization/His-containing domain of *E. coli*, EnvZ protein and the active site His was found located on a solvent-exposed helical face.

Analysis of AtHPt1 and OsHPt4 protein showed striking similarity in the secondary structure. Structure of both AtHPt1 and OsHPt4 protein showed the presence of six $\alpha$-helices similar to the Hpt protein in *Zea mays* which also found to consists of six $\alpha$-helices. The detailed analysis of structure of the AtHPt1 protein showed the presence of six $\alpha$-helices namely $\alpha1$(4-22), $\alpha2$(27-37), $\alpha3$(43-68), $\alpha4$(74-91), $\alpha5$(94-109), and $\alpha6$(112-145) while OsHPt4 protein also showed the presence of six helices, namely $\alpha1$(2-20), $\alpha2$(25-36), $\alpha3$(39-65), $\alpha4$(71-
6.3 Result and Discussion

Figure 6.15: PROSA-web analysis of modeled AtHPt1 and OsHPt4 protein confirmed the quality of the modeled structure.

88), α5(91-106), and α6(109-140) (Figure 6.16). Similar four C-terminal helices form an antiparallel four-helix bundle were observed in YPD1 (Xu & West 1999;
6.3 Result and Discussion

Figure 6.16: Cartoon representation showing secondary structures of AtHpt1 (a) and OsHpt4 protein (b).

Song et al. (1998), and the HPt domain of ArcB (Kato et al. 1997). Earlier reports have shown that the conserved histidine (His80) (Sakakibara et al. 1999) present in the center of helix D in Zea mays acts as a site for phosphorylation. The histidine residue (His74) was also found conserved in AtHpt1 at the helix D but in case of OsHpt4 conserved histidine was found replaced with glutamine residue (Glu79). Sequence similarity of specific residues surrounding the His residue has prompted postulation of structural and functional roles of the conserved residues (Xu & West 1999). It has been earlier observed that the despite the overall fold conservation, specific differences in helix length and orientation in each HPt domain may provide structural features needed for individual functions.
These structural variations likely result in modifications of surface properties designed to promote proper intermolecular contacts (Stock et al. 2000).

Figure 6.17: Electrostatic view overlapped with the cartoon secondary structure view of AtHPt1 (a) and OsHPt4 (b) protein.

Structure alignment of templates, AtHPt1 and OsHPt4 protein showed the existence of conserved residues near the active site region. With comparison to earlier resolved Hpt protein structure such as YPD1 (Xu & West 1999), the hydrophobic reverse turn between helix C and D was also found to be conserved in AtHPt1 and OsHPt4 protein structures. The Asp67 and Asp73 in helix C was found conserved in AtHPt1 and OsHPt4 and was reported earlier to serve as a hydrogen bond acceptor in other resolved Hpt proteins. Analysis of YPD1 protein showed that the conserved hydrophobic reverse turn between helices C and D and the 'N-cap' stabilization of helix C was found important for the structural integrity of the antiparallel helical motif (Xu & West 1999). The residues surrounding the active site were found to be arranged in such a manner that
6.3 Result and Discussion

it can stabilize the accessibility of His. In OsHPt4, Gly83 residue and Gly86 in AtHPt1 located four residues away from the active-site histidine residue is highly conserved amongst the known Hpt proteins. Analysis suggests that the absence of a side-chain at this position provides more exposure of the active-site histidine residue and increases the solvent accessibility of the histidine residue (Xu & West 1999). Electrostatic map analysis of the OsHpt4 proteins showed a positive patch near active site due to the presence of positively charged residues (Figure 6.17). Earlier structural analysis suggested several possible roles for positively charged residues at this location (Xu & West 1999). The structure of ArcB Hpt domain was observed to possess kinks in the helix C and helix D of the four helix bundle because of the proline residue in the middle of one helix (Xu & West 1999). Hence the structure of the Hpt domain was observed to have a concave surface surrounding the active-site histidine residue. Reports show, that this structural feature provides an extensive binding surface for the globular response regulator domains (Kato et al. 1997). Analysis of YPD1 structural domain revealed that this structural feature was not common to all the Hpt structures (Xu & West 1999). The structure of AtHPt1 and OsHPt4 proteins were also not observed to contain proline induced kinks, but was found to maintain a flat molecular surface encompassing the active-site histidine residue.

6.3.7 Binding analysis of Hpt protein with RR domain of HK in Arabidopsis and Rice

Earlier reports have established that the bacterial response regulator CheY acts as a phosphoryl donor to the yeast YPD1 protein (Janiak-Spens et al. 1999). Docking studies performed earlier shows that there can be many possible conformations for the histidine residue (His64) of YPD1 to be a reasonable distance to the active-site aspartate residue of CheY (Asp57) (Xu & West 1999). Analysis of binding site of the RR domain of AtHK1 protein and AtHPt1 protein showed residues in helix D and E of Hpt protein were found to be involved in hydrogen bond interactions. Docking analysis of RR domain of AtHK1 and AtHPt1 protein revealed the hydrogen bond interaction of Glu108, Lys104 and Cys107 of AtHPt1 protein with Ile108, Thr146 and His107 of the RR domain of AtHK1.
6.4 Conclusion

In microorganism and plants signal transduction was found to be mediated by His-Asp phosphorelay system. In His-Asp phosphorelay system, two conserved families of proteins are involved: Histidine protein kinase and Response regulator.
6.4 Conclusion

<table>
<thead>
<tr>
<th>OsHpt4</th>
<th>OsHK3b-RR</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg73 NH2</td>
<td>Lys112  O</td>
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</tr>
<tr>
<td>Gln80 NE2</td>
<td>Gln108 OE2</td>
<td>2.29</td>
</tr>
<tr>
<td>Asn105 O</td>
<td>Arg92 N</td>
<td>3.27</td>
</tr>
<tr>
<td>Tyr72 OH</td>
<td>Gln89 NE2</td>
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</tr>
<tr>
<td>Tyr72 OH</td>
<td>Gln89 OE2</td>
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</tr>
<tr>
<td>Gln69 N</td>
<td>Gln84 OE1</td>
<td>1.52</td>
</tr>
</tbody>
</table>

Table 6.6: Hydrogen bonds between modeled phosphotransfer protein (OsHpt4) and modeled His-Kinase protein (OsHK3b) from Oryza sativa. Hydrogen bond distances were calculated using HBPLUS.

Figure 6.18: Interaction of (a) AtHPt1 with AtHK1 in Arabidopsis thaliana and (b) OsHpt4 with OsHK3b in Oryza sativa. The figure was prepared using LIGPLOT.

In eukaryotes, His-Kinase proteins contain both elements of the traditional two-component pathway and thus are referred as ‘hybrid kinases’. Understanding the
structural arrangements of sensor His-Kinase domain, cytoplasmic domains and response regulator domain can assist plant biologist in understanding the fundamental mechanism of signal propagation. The conserved phosphotransfer mechanism was found common to all two-component system. Recent structural studies on various domains of His-Kinase protein have uncovered conserved residues responsible for protein-protein and protein-ligand interactions.

Figure 6.19: Schematic representation of typical hybrid type TCS hypothesized in form of predicted secondary structure of various domains in *Oryza sativa*. The phosphate is first transferred from the histidine residue in the transmitter to the aspartate residue of the attached receiver domain, then to a histidine residue on a histidine phosphotransfer (HPt) domain. Finally, the phosphate is relayed from the HPt domain to the receiver domain of a downstream response regulator protein, which results in the output response.
Analysis of CHASE domain in His-Kinase protein in *Arabidopsis thaliana* and *Oryza sativa* shows variation in the signaling domain. CHASE domain was found as an extracellular domain of 200-230 amino acids. The CHASE domain was predicted to have $\alpha+\beta$ fold, with two extended helices on both boundaries and two central helices separated by sheets. The CHASE domain were found to less conserved towards their termini while were found conserved towards the core region.

Structural analysis of cytoplasmic domain of His-Kinase proteins in *Arabidopsis* and *Oryza sativa* revealed the interaction of conserved residues with ATP and Mg$^{2+}$ ions. The cytoplasmic domain in *Arabidopsis thaliana* consist of seven $\alpha$-helix and seven $\beta$-sheets while in *Oryza sativa*, this domain consist of seven $\alpha$-helix and seven $\beta$-sheets. The residue Asp which was known to play major role in phosphorelay mechanism was found conserved in both *Oryza* and *Arabidopsis*. The interaction of ADP with conserved Asn128 was observed in both *Oryza sativa* and *Arabidopsis thaliana*. The motif G1(DxGxG$\phi$) and G2 (G$\phi$G$\phi$) was found conserved in the cytoplasmic domain in both the plant species. Structural analysis of response regulator domain of His-Kinase protein in *Arabidopsis* and *Oryza sativa* revealed conserved secondary structure motif in the domain. Analysis of HPt protein in *Oryza sativa* and *Arabidopsis thaliana* shows striking similarity in the secondary structure. Structure of HPt protein in both *Arabidopsis thaliana* and *Oryza sativa* shows the presence of six $\alpha$-helices similar to the HPt protein in *Zea mays* which also found to consists of six $\alpha$-helices. Binding analysis of the response regulator domain and HPt protein in *Oryza sativa* and *Arabidopsis* shows the interaction of the conserved secondary structure motifs and residues. The structure based approaches have proven to be viable tool to understand the complete mechanism of the any protein function. The structural analysis of various domains will be able to give a view of the structure and their interaction playing major in the phosphorelay mechanism (Figure 6.19).