Chapter 2: Review of Literature
Plants being sessile are often exploited as a source of food and shelter by a wide range of parasites including viruses, bacteria, fungi, nematodes, insects and even other plants. Some of the organisms that remain in contact may be beneficial (eg. mycorrhiza), whereas most others are pathogenic, causing diseases, resulting in death or reduced reproduction or yield (Pieterse and Dicke, 2007).

Plants however are not dormant targets to pathogen infestation. Over a period of time plants have developed an array of inducible and innate defense responses to counter the pathogen attacks. These mechanisms vary in their capacity to mount defenses against the invading pathogen. The perception of pathogen by the host is the outcome of a coordinated and sophisticated network of signaling interaction that has evolved over a period of time. Intense studies on plant defense mechanisms have taken place over the last two decades and numerous reports have been published outlining the probable defense mechanism that may be activated in the event of a pathogen attack.

2.1 Pathogen recognition and response in plants

The immunity or defense against pathogen in plants may be broadly classified into two classes: the Innate (non-adaptive immunity) and the Induced/ Effector-Triggered Immunity. In the first, conserved Pathogen-Associated Molecular Patterns (PAMPs) are recognized by cell-surface receptors, called Pattern-Recognition Receptors (PRRs), thus restricting pathogen growth. This response is also known as PAMPs Triggered Immunity (PTI) or basal resistance. Whereas in the second, plants express localized immune receptors, mostly intracellular, which can specifically recognize pathogen-derived effector molecules in a plant-cultivar and strain-specific manner, thus initiating Effector-Triggered Immunity (ETI) or gene-for-gene resistance (Chisholm et al., 2006; Jones and Dangl, 2006). These effectors are basically secreted proteins of pathogenic origin that manipulate host cell functions, whereas PAMPs are definite molecular motifs common to a wide array of pathogens, which are recognized by plants.
2.1.2 Innate / PAMPs Triggered Immunity

Pattern Recognition Receptors (PRRs) were discovered as integral plasma membrane proteins featuring extracellular LRR domains, that function by recognition of conserved Pathogen Associated Molecular Patterns (PAMPs), also called Microbe Associated Molecular Patterns (MAMPs) (Nürnberger et al., 2004; Zipfel and Felix 2005; Jones and Dangl 2006; Schwessinger and Zipfel 2008). Over the years several MAMPs originating from plant pathogens like hairpin (hrpZ), Elongation Factor Tu (EF-Tu), lipopolysaccharide, cold-shock proteins, Necrotic and ethylene- inducing peptide 1- (Nepl), flagellin chitin, ergosterol, β-glucans have been identified and characterized (Nürnberg et al., 2004; Zeidler et al., 2004; Zipfel and Felix, 2005, Qutob et al. 2006; Schwessinger and Zipfel, 2008). The recognition of different MAMPs by the specific PRRs activates defense responses that are important for host immunity to both pathogenic and non-pathogenic microbes (Akira et al. 2006; Ausubel 2005; Chisholm et al. 2006; He et al. 2007) (Figure 2.1). In addition to MAMPs/PAMPs, plants have developed mechanisms to sense infectious-self or modified-self molecules. These molecules are either from the plant itself or direct from the invading pathogen, they are called Danger-Associated Molecular Patterns (DAMPs).

The plant intracellular responses associated with PTI include rapid alteration of ion and anion fluxes across the plasma membrane, generation ROS and activation of post transcriptionally regulated Mitogen Activated Protein Kinase (MAPK) cascades. (Zipfel 2008) These changes lead to the activation of numerous genes including transcription factors, hormone-responsive proteins, RLKs, phosphatases, E3 ubiquitin ligases and defense related proteins associated with cell-wall fortifications (Asai et al., 2002; Nürnberg and Lipka 2005; Thilmony et al., 2006).

The plant cell is enveloped in the cell wall matrix, which acts as a barrier as well as a nutrient source for the potential pathogens. Pathogens capable of breaching this barrier are under molecular surveillance by the host proteins, usually receptor kinases that are localized on the cell surface or within the cytoplasm. These Receptor-like kinases (RLKs), one of the largest protein families identified in Arabidopsis thaliana (~610 members), (Shiu et al., 2004) detect the presence of MAMPs/PAMPs/DAMPs and induce PTI.
Figure 2.1. Pattern Recognition Receptors in Plants

Proteinaceous bacterial PAMPs such as flagellin and EF-Tu are recognized by the LRR-RLKs FLS2 and EFR, respectively. BAK1, a small LRR-RLK was shown to form a ligand dependent complex with FLS2 and thereby positively regulates flagellin signaling. The Arabidopsis LysM-RLK1/CERK1 is required for chitin signaling in response to fungal infection but also seems to be involved in bacterial defense reactions. Chitin binding was shown for the LysM-RLP CEBiP from Rice. The LRR-RLPs LeEIX1/2 recognizes another fungal PAMP, xylanase. Oomycetes contain β-glucans that can bind to the glucan-binding protein GBP from soybean. The LRR-RLK PEPR1 recognizes a plant-encoded wound released DAMP, AtPEP1. Theseus is a CrRLK1L protein, which monitors cell wall integrity via the perception of a yet unknown signal. Perception of the different elicitors via the specific PRRs leads to activation of innate immune reactions. LysM: lysine motif, RLP: receptor-like protein, CrRLK1L: Catharanthus roseus RLK1-like (Adapted from Postel and Kemmerling 2009)
RLKs are composed of an extracellular, a transmembrane and a cytoplasmic serine/threonine kinase domain. A common structural feature in numerous pattern recognition receptors is the leucine-rich repeat (LRR) implicated in signal perception. The best-characterized PRR in plants is the Leucine-rich repeat receptor kinase (LRR-RK), *Arabidopsis* Flagellin Sensing Receptor Kinase (FLS2), recognizing bacterial Flagellin (flg22) (Gomez-Gomez and Boller, 2000; Asai et al., 2002; Zipfel et al., 2004; Chinchilla et al., 2006). It belongs to subfamily XII of LRRRK and consists of an extracellular domain with 28 LRR motifs, a transmembrane domain, and a cytoplasmic Ser/Thr kinase domain (Gomez-Gomez and Boller, 2000). First identified in *Arabidopsis*, FLS2 orthologs have since been cloned in tomato, *Nicotiana benthamiana*, and rice (Nicaise et al., 2009). Although FLS2 directly binds flg22 and is responsible for recognition specificity, the flg22-binding site is still unknown. Nevertheless, the AtFLS2 LRR domains IX to XV contribute significantly to flg22 binding. In *Arabidopsis*, pre-treatment with flg22 restricts the growth of the pathogenic bacterium *Pseudomonas syringae* pv tomato DC3000 (Pto DC3000), and *fls2* mutants are more susceptible to this bacterium (Zipfel et al., 2004). In addition, lack of flagellin recognition allows more growth of the non-adapted bacteria *P. syringae* pv *phaseolicola* and *P. syringae* pv *tabaci* (Nicaise et al., 2009).

The elongation factor Tu (EF-Tu) acts as a very potent bacterial PAMP in *Arabidopsis* and other members of the Brassicaceae family. EF-Tu is clearly present in the secretome of several bacteria and serves as an adhesion factor at the bacterial surface, in addition to its primary role in translation (Zipfel et al., 2006). The LRR-RK EFR is the PRR for EF-Tu and belongs to subfamily XII, similar to FLS2 (Zipfel et al., 2006). The EFR structure is composed of a 21-LRR extracellular domain, a transmembrane domain, and a cytoplasmic Ser/Thr kinase domain. The autophosphorylation of EFR has been reported (Xiang et al., 2008), suggesting that it carries on active kinase domain. It has been observed in recent studies that *Arabidopsis* plants lacking EFR are more amenable to transformation by *Agrobacterium tumefaciens*, revealing that plant transformation is normally restricted by plant defenses (Zipfel et al., 2006). In addition, *efr* mutant plants are more susceptible to colonization by weakly virulent mutant strains of *Pto* DC3000 (Nicaise et al., 2009). Interestingly, the transient heterologous expression of AtEFR in *N. benthamiana*, a plant that normally lacks elf18 responsiveness, restores elf18 binding.
and responses (Zipfel et al., 2006), demonstrating that downstream signaling components are conserved between *Brassicaceae* and *Solanaceae*.

The RNA-binding motif RNP-1 of bacterial cold shock proteins (CSPs) has been found to act as a PAMP in *Solanaceae* via the recognition of the 22-amino acid core of RNP-1 (Felix and Boller, 2003). The bacterial siderophore pseudobactin is also a potential PAMP perceived by *Arabidopsis*. Very recently, bacterial non-methylated CpG DNA was also seen to be recognized as a PAMP by *Arabidopsis* (Yakushiji et al., 2009). However, the respective PRRs for all of these PAMPs are yet to be discovered.

The LRR-RK, BRI1 was classically known for its role in recognition of brassinosteroids (BRs), a class of phytohormones that control many aspects of growth and development. BRI1 was also known to form a complex with the LRR-RK SERK1, SERK3/BAK1, and SERK4/BKK1 to ensure full BR signaling. However a surprising revelation came to light when FLS2 and BAK1 were seen to interact rapidly in a ligand-dependent manner (Chinchilla et al., 2007). The rapid FLS2-BAK1 association suggested that BAK1 may exist in a preformed complex at the membrane, weakly associated with FLS2 such that, conformational changes induced by flg22 binding would result in tighter interactions, possibly due to mutual trans-phosphorylation of the kinase domains. Although BAK1 was not required for flg22 binding, early and late flg22 responses were strongly impaired in *bak1* mutants (Chinchilla et al., 2007). In *Arabidopsis* and N. benthamiana, BAK1 is also required for responses triggered by the orphan PAMPs CSP22, HrpZ, PGN, and LPS (Boller and Felix 2009). Hence it may be concluded that BAK1 is a positive PTI regulator that acts downstream of several PRRs, independently of BR. Moreover, since BAK1 is involved in PTI responses, as well as cell death control, it may be regarded as a general signaling adaptor for RKs (Boller and Felix, 2009).

In addition to PAMPs of bacterial origin, PRRs are also responsive to fungal or oomycete PAMPs. The Ethylene-Induced Xylanase (EIX) is recognized by two Receptor-Like Proteins (RLP) LeEIX1 and LeEIX2, in tomato (Ron and Avni, 2004). However, only LeEIX2 is functional and confers signaling when expressed heterologously in tobacco. Moreover, the PRR LeEIX2 triggers Hypersensitive Response (HR), which does not confirm the current understanding of PTI. In rice, Chitin, a β-1,4-linked polymer of N-acetyl-glucosamine, characteristic for fungal cell
walls, is perceived by the Chitin Oligosaccharide Elicitor-Binding Protein (CEBiP) containing two extracellular LysM domains (Kaku et al., 2006). CERK1, a RLK with three extracellular LysM domains, was recently shown to be involved in bacterial recognition, in addition to chitin responses in Arabidopsis (Miya et al., 2007). The cerk1 Arabidopsis mutants were found to be more susceptible to Pto DC3000 but were not impaired in their responsiveness to flg22, elf18, LPS, or PGN (Gimenez-Ibanez et al., 2009), suggesting that CERK1 may be involved in the recognition of yet unknown bacterial PAMP(s). Whether CERK1 is a dual-specificity PRR capable of binding different PAMPs or acts as a downstream signaling adaptor needs to be determined. In legumes, a β-Glucan Binding Protein (GBP) recognizes 1,6-β-linked and 1,3-β-branched heptaglucan (HG), which is present in the cell wall of oomycete Phytophthora sojae (Umemoto et al., 1997). Interestingly, GBP contains an intrinsic endo-1, 3-β-glucanase activity, thus potentially releasing and binding ligands concomitantly during contact with Phytophthora (Fliegmann et al., 2004).

Recent discoveries have yielded several PAMPs and their corresponding plant PRRs. Several animal PRRs have also been reported to be responsive to PAMPs like peptidoglycan-derived molecules, lipo-polysaccharides or bacterial cold shock protein, and bacterial RNA-and activate caspase1, RIP2 protein kinase, and NF-κB in the cytoplasm, (Felix and Boller, 2003; Gust et al., 2007; Silipo et al., 2008). However, the corresponding plant homolog of these PRRs remains to be isolated.

Many plant pathogens are known to produce lytic enzymes to breach the structural barriers of plant tissues. The end-products generated as a consequence of the actions of these enzymes may function as endogenous elicitors or DAMPs. Such DAMPs are typically localized in the apoplast and, as in the case of MAMPs, can serve as danger signals to induce innate immunity. In Arabidopsis, AtPepl was discovered as the C-terminal part of a small, putatively cytoplasmic protein by PROPEP1, induced by wounding, methyl-jasmonate, ethylene, flg22, and AtPepl itself. This finding indicated that AtPepl represents an endogenous signal for stress and wounding similar to systemin in tomato plants, which is released upon injury and acts as DAMP for neighboring cells. The constitutive overexpression of the PROPEP1 gene was shown to render increased resistance against Pythium spp. infection, indicating a function in innate immunity. The corresponding receptor for AtPepl was identified to be a LRR-RK called PEPR, belonging to the 28-membered
family LRR XI of *Arabidopsis* RLKs. More recently, the RLK Theseus, containing a Catharanthus roseus RLK1-like domain (CrRLK1L), that was initially shown to be involved in cell elongation control was reassigned an additional function related to defense activation in response to perception of DAMPs (Postel and Kemmerling 2009).

### 2.1.3 Induced/ Effector triggered immunity

The alternate class of immune receptors, plant Resistance (R) proteins, were discovered as polymorphic proteins characterized by the presence of conserved domains like Nucleotide Binding (NB) and Leucine Rich Repeat (LRR) domains. These proteins were reported to have the capability to directly or indirectly detect isolate specific pathogen effectors, encoded by Avirulence genes (avr) (Flor 1971; Glazebrook et al., 1997; Yang et al., 1997). Flor modeled the outcome of such plant-pathogen interactions as "gene-for-gene" mediated resistance (Flor 1971). Once activated, NB-LRRs typically induce the hypersensitive response, a form of localized Programmed Cell Death (PCD) that is thought to contribute to resistance by physically isolating the infection (Heath, 2000). The genome-wide analysis of the *Arabidopsis* genome shows that it encodes 149 predicted NB-LRRs (Meyers et al., 2003). Of these around forty R genes have been cloned over the past two decades of which majority belongs to the NB–LRR family. Structurally, NB-LRRs contain a C-terminal LRR domain, a central nucleotide-binding domain (NB), and a variable N-terminal domain (Figure 2.2).

The LRRs have a slender, arc-shaped structure with a high surface to volume ratio in relation to other globular proteins. This provides LRRs a platform for engaging in multiple physical interactions with other proteins and co-factors (Padmanabhan et al., 2008). Each LRR has a conserved core consensus of L-x-x-L-x-L-x-x-N that forms a β-strand, followed by a more variable sequence. Each repeat makes a loop that reiterates to form a stack-like super-helix, such that the β-strand aligns on one side creating a continuous β-sheet along the arc’s concave surface. The regularly spaced leucine residues, face inward to form a stable, hydrophobic core; whereas, the conserved asparagine and the variable domains make each repeat wedge-shaped, imposing the curve that is seen in all LRR structures resolved to date (Kobe and Kajava, 2001).
Figure 2.2. Schematic representation of a typical NB–LRR protein

The sub-domains are depicted as coloured boxes: CC/TIR domain (orange), NB (red), ARC1 (purple), ARC2 (blue) and LRR domain (green) whereas conserved motifs are marked as lines. The consensus sequences are written adjacent to the respective motifs. (Adapted from Lukasik and Takken 2009)
The NB domain, on the other hand, is part of a larger domain that is called the NB–ARC as it is shared between R proteins and the human apoptotic protease-activating factor 1 (APAF-1) and its \textit{Caenorhabditis elegans} homolog \textit{CED-4}. Several conserved motifs can be discerned in the NB–ARC domain that belong to the STAND (signal transduction ATPases) family of NTPases. The NACHT sister family within this class encompasses the animal NLR (NACHT-LRR/NOD-LRR) innate immune receptors, where the NB domain is also fused to an LRR domain. STAND proteins are proposed to function as molecular switches, regulating cellular responses through nucleotide dependent conformational changes (Leipe et al., 2003). The \textit{in silico} analysis and 3D modelling of NB–ARC of R proteins has revealed that they may contain three sub-domains: the NB forming a P-loop NTPase fold, the ARC1 consisting of a four-helix bundle and the ARC2 adopting a winged-helix fold (Takken et al. 2006). Most of the conserved motifs in the NB–ARC are present at the interface of these three domains where they form the nucleotide-binding pocket.

The N-terminus of NB–LRRs are structurally diverse. Whereas, some carry domains homologous to the Toll and Human Interleukin-1 receptor (TIR-1) domain, called TIR-NB–LRRs or TNLs others are referred to as CC–NB–LRRs or CNLs, as many of them contain a predicted Coiled Coil Region (CC), sometimes extended by a DNA binding domain such as a BEAF/DREAF zinc finger domain (BED) or by a Solanaceous Domain (SD) (Tameling and Takken, 2006). Plant NB-LRRs thus appear to have combined structural motifs from mammalian TLRs and intracellular Nod-like receptors (NLRs). Unlike animal innate immune receptors, plant NB-LRRs recognize specific pathogen effector proteins. Therefore, plant NB-LRRs perform a function analogous to the mammalian adaptive immune system. This implies that plant genomes must encode a large number NB-LRRs to confer resistance to many different pathogens.

The R proteins are stringently regulated, such that, they are activated during pathogen presence and inactivated in absence of pathogen effectors. Recent understanding of the R proteins reveal that inappropriate activation is prevented by auto-inhibition, which seems to be mainly accomplished by intra-molecular interaction between its various domains. Interaction and mutagenesis studies with various NB-ARC and NLR proteins, including R proteins, identified both the N-terminal part of the repeat domain and the ARC2 sub-domain to be essential for this
Figure 2.3. Model for the switch function of NBS-LRR proteins

In the absence of a pathogen, an NBS-LRR protein is in the OFF-state (resting state), in which the LRR exerts its negative role by stabilizing the ADP-bound state. The presence of an elicitor (Avr) affects the LRR domain, which induces a conformational change in the NB-ARC domain that allows the release of ADP. ATP binding subsequently triggers a second conformational change in the N-terminal effector domain, releasing its signaling potential. The ATPase activity of the protein attenuates the signaling response and returns the protein to its resting state. (Adapted from Takken et. al., 2006).
auto-inhibition (Figure 2.3). The disruption of the interaction between these two sub-domains, by mutations or domain swaps, resulted in diminished auto-inhibition and constitutive R protein activation (Rairdan and Moffett 2007). Furthermore, various studies have shown that the C-terminal part of the LRR domain provides pathogen recognition specificity (Takken et al., 2006; Rairdan and Moffett 2007). Hence, the LRR domain has a dual function as it provides auto-inhibition and translates pathogen recognition into activation. Recent reports on the tomato R protein 1-2 revealed that it tightly binds ADP in vitro and that mutations reducing its ATP-hydrolysis rate result in constitutive defense activation. On the basis of these data, it was proposed that R proteins function as nucleotide-controlled molecular switches. In this model, the ADP-bound state represents the “OFF” state and the ATP-bound state the “ON” state of the protein (Figure 2.3). The recognition of an effector triggers a conformational change that results in an “intermediate” open state, which enables ADP to be exchanged for ATP. Upon ATP-binding, the R protein adopts its active conformation (“ON” state) that subsequently unchains, in a still unknown way, host defenses. ATP hydrolysis eventually returns the protein to its auto-inhibited “OFF” state. (Takken et al., 2006)

The exact mechanism of recognition of pathogen effectors by the LRR domains still remains unclear. Some NB–LRR proteins have been shown to bind to their cognate AVRs directly, whereas others have been shown to interact indirectly through an intermediary host-factor (Caplan et al., 2008). Such a host-factor could either represent a virulence target of the effector [Guard Model] (van der Biezen and Jones, 1998) or a target mimic [Decoy Model] (van der Hoom and Kamoun, 2008). The Guard Model predicts that R proteins act by monitoring (guarding) the effector target and that modification of this target by the effector results in the activation of the R protein, which triggers disease resistance in the host (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). The indirect effector perception mechanism outlined by the Guard Model explains how multiple effectors could be perceived by a single R protein, thus enabling a relatively small R gene repertoire to target the broad diversity of pathogens that attack plants (Dangl and Jones, 2001). It is also evident from the Guard Model that the guarded effector target (also called the Guardee) is indispensible for the virulence function of the effector protein in the absence of the cognate R protein.
The classical Guard Model (A) is contrasted with a modified Guard Model in which the effector targets multiple plant proteins (B) and the Decoy Model (C). Effectors are depicted in gray, operative effector targets in purple, effector target (guardee) in green, decoy in blue, and the R protein in orange (Adapted from van der Hoorn and Kamoun 2008).
However, recent evidences on indirectly recognized effectors have emerged that are not consistent with the original description of the Guard Model. It has now been reported that many pathogen effectors have multiple targets in the host and that classical guardee proteins are not essential for the virulence activities of effectors in plants lacking the R protein. The new revelations on additional targets like AvrPto (Xiang et al., 2008), Avr2 (Shabab et al., 2008) etc. has prompted that some host targets of effectors may act as decoys to detect pathogen effectors via R proteins. Besides, from the evolutionary point of view; the Guard theory seems to be an unstable proposition since it is subject to two opposing forces of natural selection in plant populations where R genes are polymorphic. The first arises out of the natural selection driving the pathogen effectors to decrease its binding efficacy with the host effector targets (guardee proteins), in absence of a functional R protein, so as to avoid immune detection by the host. The second, contradicting force arises out of the natural selection pressure on the host to bind the pathogen effectors with increased efficiency, in presence of a functional R protein, so as to enhance pathogen perception and subsequent defense. As both these opposing forces converge on the effector-guardee interactions, leading to an unstable scenario, it was suggested that another set of host proteins, “Decoy”, may be the actual target of the effector molecules (van der Hoorn and Kamoun 2008). This new form of interaction, known as the Decoy Model, was based on recent understanding of the behavior of well characterized pathogen effectors like AvrPto, AvrBs3, Avr2 and AvrRpt2, and their putative decoys Pto, pBs3, RCR3 and RIN4 (Zhou and Chai, 2008; Zipfel and Rathjen, 2008). The decoy proteins themselves have no function either in the development of disease or resistance but they simply mimic effector targets to trap the pathogen into a recognition event. The Decoy Model is distinct from the classical and refined Guard Models that imply that the manipulation of the guarded effector target by the effector benefits pathogen fitness in the absence of the R protein (Figure 2.4).

However, in both these models the modification of the host target by the effector triggers defense in resistant plants. Either way, the R protein activates defense signaling after AVR recognition whereby the R protein acts as a molecular switch.
2.1.4. Recent insights on Innate and Induced defense responses in plants

The first level of microbe recognition is performed by the membrane proteins termed pattern recognition PPRs is common to all multicellular organisms and leads to an array of defense responses and redeployment of cellular energy in a fast, efficient, and multi-responsive manner, that prevents further pathogen ingress. PAMP recognition leads to a chain of signaling events broadly referred to as general defense responses in plants and also results in plant systemic acquired resistance (Mishina and Zeier 2007). Due to the onslaught of PTI, successful pathogens evolved secreted effectors targeting key PTI factors to interfere with plant defense. In turn, some plant cultivars have co-evolved resistance (R) proteins to directly or indirectly detect these effectors (previously termed avirulence or Avr proteins) according to the Gene-for-Gene Theory and leading to the ETI, which is often accompanied by the hypersensitive response, a form of programmed cell death (Chisholm et al. 2006; Jones and Dangl 2006).

The hallmark of PRRs is their sensitivity and specificity to the diverse array of MAMPs/PAMPs that can be detected even at sub-nanomolar concentrations. However, plants lacking these PRRs are completely blind to the onslaught of the corresponding pathogen effectors. A given PAMP/MAMP is recognized through a specific conserved epitope, such as the stretch of 22 amino acids (flg22) in the N terminus of flagellin. Recent discoveries have indicated that pathogens have evolved to develop strategies to avoid perception by the PRRs. It has been seen that the introduction of mutations into the amino acid sequence of flagellin proteins derived form Pseudomonas syringae pv. tabaci 6605 and flg22Pa makes the molecule unrecognizable by plant PPR, FLS2. But it also renders the microbe motionless and reduces its virulence (Naito 2009). Thus, the specificity of the PRR appears to be focused exactly on a highly conserved domain of the MAMP that is functionally important to the microbe. Hence, the process of recognition of flagellin through FLS2 homologs is a primitive phenomenon, which appearars conserved, in higher plants.

Another well-characterized PRR of Arabidopsis, EFR appears to be confined to the Brassicaceae and is not found in other dicots or monocots. This suggests that EF-Tu perception is a relatively newer evolutionarily development when compared to flg22 perception. However, the genome analysis of the different plant genomes sequenced so far shows the presence of homologs of this EFR-encoding gene, with a
comparable LRR structure. The rice genome is known to encode about 40 such homologs, of which Xa21 is reported for detection of the Avr gene AvrXa21 (Song et al., 1995). Xa21 belongs to subfamily XII of the LRR-RKs and is highly similar to EFR and FLS2. Xa21 possesses a non-RD kinase, whose presence may be correlated with a role in innate immunity across kingdoms (Dardick and Ronald 2006). Although the identity of AvrXa21 was until now unknown, recent work identified AvrXa21 as a type I secreted sulfated peptide (Nicaise et al., 2009).

2.1.5 Modulation of plant defense responses by Pathogen Effectors

Plant mutants lacking components of the PTI pathway have been observed with a compromised immunity against pathogens, in contrast to their natural counterparts (Boller and Felix 2009). Hence the role of PTI in offering the first line of defense against pathogens cannot be underestimated. Additionally, a diverse array of bacterial virulence factors, including the phytotoxin coronatine, extracellular polysaccharides, and proteinaceous effectors secreted through the type III secretion system (TTSS), in bacteria are now known to target and suppress PTI in their hosts. This has emerged as an evolutionary adaptation of the pathogens against the innate plant defenses. Among the various effectors secreted to mitigate plant defenses, two effectors, AvrPto and AvrPtoB (from P. syringae strain DC3000), were seen to physically interact with the kinase domains of PRRs like FLS2, EFR or BAK1 such that these physical interactions inhibit their kinase activity (Xiang et al., 2008). AvrPto appears to be a novel protein, whereas, AvrPtoB contains a C-terminal domain that resembles E3 ubiquitin ligase. The ubiquitination by this domain initiates the degradation of a tomato kinase (Fen) that is part of a unique and presumably ancient ETI pathway. The same domain is also responsible for the degradation of other PRRs, and thus its more decisive role may be to impede PTI responses (Rosebrock et al. 2007). The ability of AvrPto and AvrPtoB to derail PRRs provides a satisfying explanation for previous discoveries that these effectors could suppress a variety of responses of PTI, including callose deposition, activation of kinase cascades, and expression of MAMP-responsive proteins and small RNAs (Navarro et al. 2008).

However, not all bacteria, and not all strains of P. syringae, express AvrPto and AvrPtoB, which suggests the existence of other strategies to inhibit PRR
signaling. The phosphothreonine lyase, HopAII, present in many if not all *P. syringae* strains, has been seen to dephosphorylate Mitogen-Activated Protein Kinases, implicated in plant innate responses. This gives an insight into the fact that pathogens have evolved to target the PTI defense at various stages to effectively evade detection and establish colonization in hosts.

In addition to bacterial pathogens, phyto-pathogenic fungi and oomycetes also target the PTI responses like cell death, in ways to assist their colonization in hosts, and these strategies provide interesting contrasts to mechanisms seen in bacterial pathogens. (Schulze-Lefert and Panstruga 2003) The biotrophic fungi, *Blumeria graminis* f. sp. *hordei* (Bgh) provided early evidences that fungi suppress cell death in plants during infection. During infection, these fungi were seen to cause the ‘Green-Island’ effect wherein, the leaf tissue adjacent to the fungal infection was kept alive while the surrounding tissue underwent senescence. The ability of the Powdery Mildew pathogen, *Blumeria graminis* to penetrate its host, Barley and sustain infectious growth has been attributed to the suppression of cell death by plant genes such as MLO and BI-1 (Huckelhoven et al., 2003). The alteration of sequences by point mutations, frame-shifts, gene deletions, and transposon insertions in pathogen effector genes, is now seen as a fast emerging mechanism in pathogens to evade ETI. A good example of such a strategy in fungal pathogens can be seen in almost all sequence variants described for the chitin-binding Avr4 effector protein of *Cladosporium fulvum*, which showed single amino acid substitutions that abolish recognition by the cognate Cf-4 protein but retain their ability to bind to chitin (Stergiopoulos et al. 2007).

### 2.2 Signaling pathways in Plant Defense

Plants and animals differ strongly in their fundamental biological processes, which bring about major deviations in their capacity and strategy to defend themselves against potential pathogens. Whereas plants being sessile require its pathogen to be mobile to spread infection, animals being mobile are able to spread infections by contact. Additionally, animals have a circulatory system that enables the delivery of somatically generated, adaptive immune responses to infection sites, a component that plants lack. Plant cells are fortified with a cell wall that provides an
effective barrier against potential pathogen attack, which animal cells lack. Regardless of these obvious differences, plant disease resistance and mammalian innate immunity share several remarkable similarities at the molecular level. The study of the mechanisms of perception of pathogen invasion and the corresponding host signaling events in response, reveal striking similarities between plants and animals. These observations highlight the presence of a conserved defense-related signaling system in diverse kingdoms, throughout evolutionary processes, that may have led to the evolution of the innate immunity system (Dangl and Jones 2001; Nurnberger et al. 2004).

The early events in innate signaling such as protein phosphorylation or the activation of plasma membrane proteins stands to mobilize directly or indirectly, diverse secondary signaling molecules, like phytohormones, which regulate many downstream processes. Phytohormones are generally small molecules that are essential for the regulation of plant growth, development, reproduction and survival, wherein they act as signal molecules and occur in low concentrations. Classical phytohormones are Abscisic acid (ABA) and Ethylene (ET), however, smaller signaling molecules such as, jasmonates (JAs) and salicylic acid (SA) are also recognized as phytohormones. Investigation of different plant–pathogen interaction systems have also demonstrated the ability of plants to activate distinct defense pathways involving different downstream regulators and signaling molecules, based on the specificity of the pathogen.

2.2.1 Oxidative Burst and generation of Active Oxygen Species

The recognition of pathogen attack in plants is known to activate the production of Reactive Oxygen Species (ROS), through a mechanism commonly known as Oxidative Burst. The generation of superoxide anion ($\text{O}_2^-$), hydroperoxyl radical ($\text{HO}_2^-$), or its dismutation product hydrogen peroxide ($\text{H}_2\text{O}_2$) in the apoplast, has been documented following recognition of a variety of pathogens. R-gene mediated recognition of pathogens by the plant immune system also elicits a biphasic ROS accumulation with a low-amplitude, transient first phase, followed by a sustained phase of much higher magnitude correlating with disease resistance (Lamb and Dixon, 1997). However, virulent pathogens that evade host recognition induce
only the transient, low-amplitude first phase of this response, suggesting a role for ROS in the establishment of defense responses. Microbe elicitors, PAMPs and MAMPs are also known to trigger oxidative burst. The ROS produced during pathogen challenge are largely derived from the activity of membrane-localized NADPH oxidases and Respiratory Burst Oxidase Homologs, (Rboh) (Torres et al., 2006), with AtRbohD being the most important for PAMP-triggered oxidative burst (Nuhse et al., 2007; Zhang et al., 2007). The relative position of oxidative burst in the sequence of signaling events during PTI is still not clear. In Arabidopsis, RbohD-dependent ROS production is suggested to be downstream or independent of MAPK cascade activation (Zhang et al., 2007).

In the plant cell, ROS can also lead to fortification of host cell walls via cross-linking of glycoproteins (Bradley et al., 1992; Lamb and Dixon, 1997), or lipid peroxidation and membrane damage (Lamb and Dixon, 1997; Montillet et al., 2005). However, ROS are important signals mediating defense gene activation in plants (Levine et al., 1994). Although ROS is normally correlated with the successful disease resistance responses, some pathogens may exploit the production of ROS to their own advantage. Specifically, fungal necrotrophs appear to stimulate ROS production in the infected tissue to induce cell death that facilitates subsequent infection (Govrin and Levine, 2000). The fungal necrotroph triggers significant changes in the peroxisomal antioxidant system, leading to a collapse of the protective mechanism at advanced stages of infection. This process is partly related to senescence (Kuzniak and Sklodowska, 2005). Also, some pathogens interfere with the chlorophyll degradation pathway which results in accumulation of ROS thereby increase host susceptibility (Kariola et al., 2005). Thus, ROS is produced as part of a complex network of signals that respond to pathogen attack and mediate multiple responses, sometimes with opposite effects, in different contexts or in response to different pathogens.

ROS was believed to act synergistically in a signal amplification loop with SA to drive the HR and the establishment of systemic defenses concurrent with the rise in endogenous levels of SA in cells surrounding the infection sites (Enyedi et al., 1992). Evidences also emerged showing the down-regulation of ROS scavenging systems in response to SA accumulation, further supporting the hypothesis. (Klessig et al. 2000). However, ROS and SA were actually seen to antagonize each other's action in the
regulation of cell death expansion at the margins of pathogen-triggered HR lesions as was evident from the lesion mimic mutant *lsd1* (Torres et al., 2005). *lsd1* was unable to retain the initial HR induction following pathogen perception. Interestingly, the ROS produced by *AtrbohD* and *AtrbohF* were seen as negative regulators of the unrestricted cell death expanding from the margins of an initial HR site in *lsd1*, whereas SA produced through Isochorismate synthase is recognized positive regulator of this cell death in plants (Torres et al., 2005). The synergistic effect of SA and JA has also been seen drive ROS production and corresponding cell death (Mur et al., 2006).

ROS signaling is said to work in conjunction with NO in the potentiation of the pathogen-induced cell death (Delledonne et al., 2001). Cytological studies have shown that ROS and NO are associated with cell death adjacent to infected cells and that both signals modulate each other's accumulation (Tada et al., 2004). Interestingly, both ROS and NO are also seen to collaborate in the abscisic acid (ABA)-induced stomatal closure (Desikan et al., 2004). Responses associated with ROS have also been seen to interact with ethylene signaling pathways as Ethylene has been seen to induce programmed cell death and senescence (de Jong et al., 2002). Both ROS and ethylene have been implicated in signaling in response to viral infection (Love et al., 2005). Interestingly, the ethylene receptor ETR1 can function as an ROS sensor, mediating stomatal closure in response to H$_2$O$_2$ (Desikan et al., 2005). Thus, ROS signaling interacts with several regulatory events in a complex network of signals that govern the response to pathogens and other factors of the environment as well as developmental cues.

### 2.2.2. Role of Salicylic Acid in defense signaling

SA is one of a wide variety of phenolic compounds bearing a hydroxyl group or its derivative that are synthesized by plants. SA in plants can be generated via two distinct enzymatic pathways that require the primary metabolite chorismate. Chorismate-derived L-phenylalanine (ICS1) can be converted into SA via either benzoate intermediates or coumaric acid via a series of enzymatic reactions initially catalyzed by Phenylalanine Ammonia Lyase (PAL). However, bulk of the SA synthesized in response to pathogen perception is via the conversion of Chorismate...
through two-step process involving Isochorismate Synthase (ICS) and Isochorismate Pyruvate Lyase (IPL).

The possible role of SA in signaling disease resistance was discovered when tobacco leaves administered with SA derivatives showed PR protein accumulation and enhanced resistance to tobacco mosaic virus (TMV) infection. (Volt et al. 2009). Subsequently, SA treatment was to induce PR gene expression and resistance to viral, bacterial, and fungal pathogens in many plant species. These evidences were further validated by the analysis of mutants form tobacco and Arabidopsis. Transgenic plants expressing nahG and having an impaired SA biosynthetic pathway also have a diminished ability to accumulate endogenous SA and elicit the systemic expression of PR genes, during pathogen infestation. Instead, they display a heightened susceptibility to both virulent and avirulent pathogens (Delaney et al. 1994). Thus, the increase in the endogenous levels of SA and its conjugates in pathogen-inoculated plants coincides with the elevated expression of genes encoding the pathogenesis-related (PR) proteins and the activation of disease resistance. The prevention of SA accumulation, by its degradation by transgenic plants that expressing NahG or by blocking SA synthesis effectively blocks the activation of SA-dependent defense responses. The disease resistance in Arabidopsis thaliana is affected through two pathways: Non-Expressor of Prl (NPR1) gene, also called Non-Inducible Immunity1 (NIM1), an important component of SA signaling and the NPR1-independent pathway.

The overexpression of NPR1 enhances resistance in Arabidopsis and rice. NPR1 contains an ankyrin-repeat domain, which in other proteins mediates protein–protein interaction and a BTB/POZ domain. The protein occurs in the nucleus, where it functions in SA-mediated PR-1 gene induction, and in the cytosol, where it plays a role in antagonistic cross talk between SA and JA (Volt et al., 2009). Members of the TGA-element binding protein (TGA) family of basic-leucine-zipper (bZIP) DNA-binding proteins interact physically with NPR1 in yeast two-hybrid assays. NPR1 interacts with five different Arabidopsis TGA factors in yeast and the interaction between NPR1 and TGA1 or TGA4 was detected only upon SA treatment of leaves in Arabidopsis (Kesarwani et al. 2007). This interaction depended on SA-induced changes to the redox environment that resulted in the reduction of two cysteine residues that are conserved inTGA1andTGA4 (Durrant and Dong, 2004). In
Arabidopsis, the interaction between TGA2 and NPR1 can be detected in the absence of SA, but is enhanced by SA treatment of leaves, whereas the ability of TGA2 and TGA3 to activate transcription requires SA and NPR1 (Durrant and Dong, 2004). Since the NIMIN1 can form a complex with TGA2 or TGA6, NPR1, and a PR-1 promoter element in yeast, it might modulate TGA-dependent transcriptional activation of SA-regulated genes.

The ETI response in Arabidopsis against Turnip crinkle virus and Cucumber mosaic virus Y are conferred by the HR to Turnip Crinkle Virus (HRT) and Resistance Gene Cucumber Mosaic Virus Y (RCY1) genes, suggested the involvement of an NPR1-independent, SA-dependent defense mechanism in some plant–pathogen interactions. This resistance was compromised in NahG plants, but not in the npr1 mutants (Shah, 2003). Similarly, NahG but not npr1 effectively suppressed R-gene mediated resistance to various Pseudomonas syringae strains and Peronospora parasitica biotypes. The existence of an NPR1-independent mechanism is also supported by studies of various Arabidopsis constitutive-defense-signaling mutants. The Arabidopsis suppressor of SA-insensitivity2 (ssi2) mutant allele that confers enhanced resistance to Pseudomonas syringae and Peronospora parasitica is compromised in eds5 and NahG mutants but is retained in the npr1 mutant background. The NPR1-independent, SA-dependent defense mechanism is also activated in the Arabidopsis ssi1, constitutive expressor of PR genes5 (cpr5), cpr6 and hypersensitive response like lesions1 (hrl1) mutants (Shah 2003). It has been suggested that the NPR1-independent mechanism that is activated in cpr5 and cpr6 is akin to the local resistance activated in a leaf that is challenged with an avirulent pathogen (Clarke et al., 2000).

2.2.3. Role of Jasmonic Acid in defense signaling

Oxylipins are bioactive signaling molecules derived from oxygenated polyunsaturated fatty acids ubiquitously found in most living organisms. The oxylipin Jasmonic acid (JA) regulates many aspects of growth, development, and environmental responses in plants, particularly defense responses against herbivores and necrotrophic pathogens. The mechanical damage to plant tissue is sufficient to trigger a rapid and transient jasmonoyl-isoleucine (JA/JA-Ile) burst in plants.
Additionally, many wound-induced defense responses are stimulated by compounds in insect oral secretions and by host-derived elicitors. (Stork et al., 2009). Several chemical elicitors, including the peptide systemin and cell wall-derived oligosaccharides, also induce synthesis of the hormone in undamaged leaves (Boller and He 2009). It has thus been suggested that cell surface receptors or other sensory systems exist that, upon recognition of non-self or damage-associated (self) signals, activate the octadecanoid pathway for JA synthesis. This concept is consistent with models of plant innate immunity in which pathogen- or microbe associated molecular patterns (PAMPs/MAMPs) trigger host defense responses by activating pattern recognition receptors (PRRs) at the plant cell surface (Boller and He 2009).

The pathway for JA synthesis from \( \alpha \)-linolenic acid (18:3) was first proposed by Vick & Zimmerman and has been subsequently reviewed in due course of time. Mutants of *Arabidopsis* helped researchers define the biochemical pathway for synthesis of JA-Ile, the active form of JA hormone, and demonstrated that JA is required for plant survival of insect and pathogen attacks and for plant fertility (Browse, 2009). Following synthesis, putative receptor proteins perceive JAs, which presumably activates a signal transduction pathway culminating in the transcriptional activation or repression of a large number of JA-responsive genes. JAs are known to inhibit root elongation, and this property has been extensively exploited for the identification of JA signaling mutants.

One of the first JA signaling mutants identified was the *Arabidopsis coronatine insensitive1* (*coil*) mutant. Root elongation of *coil* mutant seedlings showed reduced sensitivity to JAs but also to coronatine (COR), a functional JA homolog and toxin produced by the bacterial pathogen *Pseudomonas syringae* (Feys et al. 1994). The *coil* mutant displays defects in many JA-dependent functions, such as fertility, secondary metabolite biosynthesis, pest and pathogen resistance, and wound responses. Two other JA signaling loci, *JAR1* (*JASMONATE RESISTANT1*) and *JIN1* (*JASMONATE INSENSITIVE1*), were identified from analyses of the *Arabidopsis jar1* and *jin1* mutants, which also show reduced sensitivity to exogenous JAs. *JAR1* encodes a JA amino acid synthetase involved in conjugating jasmonic acid to Ile (Staswick and Tiryaki, 2004). *JIN1* (also known as MYC2) encodes a basic helix-loop-helix-type transcription factor involved in the transcriptional regulation of JA-responsive gene expression (Lorenzo et al. 2004). The recent cloning of the *JAI3*
(JASMONATE INSENSITIVE3) locus (Chini et al. 2007) has mechanistically linked the functions of COII, JARI, and JINI. Further, in normal cells, having low JA levels, transcription factors that promote expression of JA-responsive genes are repressed by members of the JASMONATE ZIM-domain (JAZ) protein family (Chini et al. 2007; Thines et al. 2007). The increased JA levels were shown to stimulate JAZ binding to COII, which is the F-box protein component of the E3 ubiquitin ligase SCFCOII (Balbi and Devoto, 2008; Thines et al. 2007). The hormone-induced COII-JAZ interaction triggers JAZ degradation via the ubiquitin/26S proteasome pathway, thereby releasing transcription factors from repression. Recent evidence indicates that the repressive action of JAZ proteins depends on their ability to dimerize via the conserved ZIM-domain (Chini et al. 2009; Chung and Howe, 2009).

In addition to the aforementioned components, the SCFCOII complex acts as a key component of most or all genetically defined jasmonate responses. The determininistic feature of this complex is COII, may function as E3-type ubiquitin ligases by physically associating with Skp-like proteins, cullin and Arabidopsis thaliana RING-box1 (AtRbx1) to form active SCFCOII complexes (Xu et al. 2002). Thus once activated by jasmonates, SCFCOII acts as the central regulator in the pathway determining whether regulatory proteins are to be modified or degraded by the proteolytic pathway, post ubiquitination.

2.2.4. Role of Ethylene in defense signaling

Ethylene (ET) is a simple gaseous olefin that is known to regulate diverse aspects of the plant life cycle like seed germination, root initiation, root hair development, flower development, sex determination, fruit ripening and senescence. However, recent evidences also implicate it in to biotic (such as pathogen attack) and abiotic (such as wounding, hypoxia, and chilling) stresses. Ethylene has been shown to act in conjunction with the JA-signaling pathway to mediate defense responses against pathogen attack. The factor connecting these two signaling pathways is the transcription factor Ethylene Response Factor1 (ERF1) (Lorenzo et al. 2003). ERF transcription factors are a subfamily of the APETELA2 (AP2) transcription factor family that contains a single DNA-binding domain. The target sequence for ERF transcription factors (TF) is the GCC box that is found in several promoters of PR genes as well as ET and JA-inducible genes (Gutterson and Reuber 2004).
The transcriptomic analysis of 1,534 TFs from Arabidopsis following inoculation with the incompatible necrotrophic pathogen Alternaria brassicicola or treatment with methyl jasmonate (MeJA) identified 20 TFs commonly induced by both treatments (McGrath et al. 2005). Of these, two genes belonging to the AP2/ERF family, AtERF4 and AtERF2, revealed an antagonistic relationship. Whereas AtERF4 acted as a negative regulator of JA-responsive defense gene expression and resistance to the necrotrophic fungal pathogen Fusarium oxysporum, AtERF2 acted as a positive regulator. Further the role of these two gene products was determined in Arabidopsis plants either overexpressing the TFs or with a T-DNA insertion in the gene. In the case of AtERF2, its role as a positive regulator of MeJA response was confirmed and additionally, a reduction in disease symptoms relative to wild type after inoculation with F. oxysporum was observed, further supporting a role for AtERF2 as a positive regulator of disease resistance. The analysis of AtERF4 involvement in defense gene regulation showed a lower induction of two genes PDF1.2 and CHIB, known to be regulated by the JA pathway, in overexpressing lines, compared to wild-type plants treated with MeJA. However, an increase in basal transcript levels was observed in the T-DNA lines that did not express AtERF4 and overexpressing plants, when challenged with F. oxysporum, exhibited greater disease symptoms than the wild type. Together, these results strongly suggested the role of AtERF4 as a negative regulator of both JA-dependent response and resistance to necrotrophic pathogens (McGrath et al. 2005). Another pathogen-induced ERF gene, TaERF3, isolated from wheat showing low sequence similarity to other known ERFs, but highly conserved DNA-binding domains (Zhang et al., 2007), suggested it to be a new member of the ERF family. The TaERF3-GFP fusion product showed it to be nuclear localized and was found to be rapidly induced on treatments with SA, JA, or ET as compared to pathogen exposure. Similarly, transgenic expression of another ERF-type TF, HvRAF, from barley (Hordeum vulgare) in Arabidopsis provided increased resistance against the bacterial pathogen Ralstonia solanaceaeum (Jung et al. 2007). Thereby it can be said that ERFs are active components of the JA-Ethylene defense signaling pathways in diverse plant species.
2.2.5. Role of Abscisic Acid in defense signaling

The phytohormone Abscisic acid (ABA) was first discovered in young cotton fruits and sycamore leaves as dormin or abscissin. The most documented roles of ABA have been in seed maturation processes, acquisition of desiccation tolerance and dormancy. During vegetative growth, ABA has been recognized as the key hormone that confers tolerance to environmental stresses, most importantly drought and high salinity, thereby permitting plants to colonize ecological niches where water availability is limited. Even though it has mostly been studied in the context of stress signaling during vegetative growth, ABA has been linked to pathogen susceptibility in the recent times (Anderson et al. 2004). The advent whole genome approaches to study gene expression profiles has revealed a significant overlap between the gene activities influenced by ABA and type III virulence factors, such as those from the biotroph *Pseudomonas syringae* DC3000 (de Torres-Zabala et al. 2007). Exogenous application of ABA has been shown to promote colonization in both virulent (DC3000) and non-virulent (*hrpA*) *P. syringae*. Consequently it may be inferred that ABA negatively regulates post-invasion pathogen immunity. Supporting this argument is the fact that reducing the synthesis and sensitivity of the plant to ABA, either by mutations (such as *abil-1* and *abi2-1*) or over-expression of relevant genes like 35S::*HAB1*, leads to a 20–80-fold increase in pathogen resistance as compared to that of wild-type plants. Conversely, mutants those are hypersensitive to ABA support an average multiplication of 30-fold more *P. syringae* DC3000. However, the role of ABA appears to be more complex, as *Arabidopsis* mutants, deficient in ABA are more sensitive to infection by the fungal pathogens like *Pythium irregulare* (Adie et al. 2007) and *Leptosphaeria maculans* (Kaliff et al. 2007). In these two cases, ABA thus appears to have a positive role in activating the pathogen defense system. Further complexity arises when pathogens are tested on ABA signaling mutants, such as *abi4*, which displays opposite resistance responses towards *Pythium irregulare* and *Leptosphaeria maculans*. Similarly, the mutations *abil-1* and *abi2-1* actually foster differential resistance responses against the same pathogen, *L. maculans* (Kaliff et al. 2007). The transcriptome and meta-analysis of expression profiles altered by infection with the necrotroph *Pythium irregulare* identified many JA-induced genes and also highlighted the importance of ABA as a regulator as the ABA Responsive Element (ABRE) appears in the promoters of many of the defense genes (Adie et al. 2007).
is very likely that the final response output to a particular pathogen may be influenced by mutually synergistic or antagonistic interactions with other hormones (Anderson et al. 2004). There are other possible connections between pathogen resistance and ABA signaling, including the fact that both pathogen attack and ABA trigger the formation of the secondary messenger hydrogen peroxide which is dependent on the NADPH oxidases \textit{RbohD} and \textit{RbohF} (Kwak et al. 2003; Torres and Dangl 2005). These molecules are known to participate in the regulation of the stomatal pore through several input pathways, including that of ABA. Recent studies suggest that the stomatal pore is a critical component of the innate immunity defense against pathogen invasion (Melotto et al. 2006). \textit{P. syringae} mutants defective in producing coronatine and its precursor 12-oxo-phytodienoic acid, fail to cause disease when deposited on the leaf surface, but remain virulent when delivered directly into the leaves by injection. It is also reported that virulent \textit{P. syringae} moves towards open stomata when inoculated at the leaf surface, and although the deposition of the bacteria provokes rapid closure of the stomata, they re-open within 3 hours (Melotto et al. 2006). Mutant bacteria lacking coronatine fail to colonize the leaf interior, which correlates with the failure to re-open stomatal apertures. The initial rapid stomatal closing providing pre-invasive immunity is thereby countered by coronatine. Mutant plants deficient in FLS2 seem to be more susceptible to infection by \textit{P. syringae}. The flg22 peptide alone can also trigger stomatal closure in wildtype \textit{Arabidopsis}, suggesting that its corresponding receptor is implicated in the stomatal response to pathogen invasion. The stomata of mutant \textit{ostl} fail to close upon treatment with flg22 and these mutants also support the multiplication of \textit{P. syringae}, regardless of whether it does or does not produce coronatine (Melotto et al. 2006). Thus, the ABA signaling pathway in stomatal closure regulated by \textit{OST1}, which acts upstream of hydrogen peroxide production (Mustilli et al. 2002), and the immune response pathway dependent on FLS2 are interconnected. These observations argue that the stomata are not passive ports of bacterial entry, but are part of the innate immunity defense involving the \textit{OST1} kinase that acts as a barrier against bacterial infection.
2.2.6. Role of Nitric oxide in defense signaling

Nitric oxide (NO) is a small gaseous radical with a wide array of signaling functions attributed to it. In plants, NO was first observed to play a deterministic role in mediating defense reactions against bacterial pathogens (Delledonne et. al. 1998). Later, it was also seen to influence numerous physiological processes like germination, leaf expansion, lateral root development, flowering, stomatal closure, cell death and defense against biotic and abiotic stresses (Wilson, 2008). Despite mounting evidences of the role of NO in plant defense responses, the pathways leading to NO signaling remains to be elucidated. NO-related signaling can be attributed to the various NO derivatives, collectively referred to as Reactive Nitrogen Species (RNS). RNS comprise of the NO radical (NO'), its nitroxyl (NO') and nitrosonium (NO*) ions, peroxynitrite (ONOO'), S-nitrosothiols and higher oxides of nitrogen and dinitrosyl–iron complexes.

RNS has been seen to interact with the amino acids or prosthetic groups of bioactive proteins and consequently modifying them. More specifically, the major NO-associated protein modifications are the covalent modifications of cysteine (S-nitrosylation). S-nitrosylation refers to the covalent attachment of an NO moiety to the thiol side chain of cysteine and has been implicated in gene regulation. NPR1, a key component of systemic disease resistance and signal cross-talk, was seen to be regulated via S-nitrosylation (Tada et al. 2008). The S-nitrosylation facilitates oligomerisation of NPR1, whereas thioredoxins support reductive monomer release, a reaction that is boosted by Salicylic acid (SA). NPR1 is also known to suppress JA responses in the cytosol, in addition to the induction of systemic defense. (Spoel et al. 2003) Thus, S-nitrosylation is thought to be the contributing factor in the negative cross talk between Salicylic acid (SA) and Jasmonic Acid (JA) signaling pathways. Moreover, a JA biosynthetic enzyme, allene oxide cyclase, has been shown to be S-nitrosylated during the hypersensitive response, which brings to light another mechanism regulating oxylipin levels (Romero-Puertas et al. 2008). Besides its apparent influence on SA/JA cross-talk, NO also affects ethylene, abscisic acid (ABA) and auxin signaling. S-nitrosylation of ethylene biosynthesis enzyme, methionine adenosyltransferase 1 (MAT1) inhibits ethylene production (Lindermayr et al. 2006), whereas NO is required for downstream responses to auxin and ABA (Melotto et al. 2006; Correa-Aragunde et al. 2004). The cross talk between different
phytohormones and NO during innate immune reactions is an outstanding example of the emerging mutual influences and the regulatory role of NO in vital functions of plants. NO mediates the ABA-dependent and SA-dependent stomatal closure upon pathogen recognition. But, Coronatine, a functional mimic of JA, has been seen to reverse this effect and lead to stomatal reopening. These data implicate the involvement of at least three phytohormones in pathogen-induced stomatal movements and suggest NO as a key mediator of these hormone responses (Melotto et al. 2006). Although the role of NO in stomatal closure remains to be defined, S-nitrosylation of K\(^+\) outward rectifying channels supports a potential role of this post-translational modification in modulating stomatal movements (Sokolovski et al. 2006).

2.3 Networking of hormone signaling pathways in plant defense

Unlike animal, plants do not have specialized tissues or cells to carry out immune functions, when attacked by pathogens. Consequently, plants undergo cellular reprogramming to prioritize defense over their normal cellular functions when challenged by a potential pest or pathogen. Oxidative burst, following the generation of ROS, thereby leading to localized programmed cell death at the site of invasion is the first innate response in defense against biotrophic pathogens and sucking insects, which rely on living host cells to provide nutrients. However, this strategy is exploited by cell necrotrophs for their proliferation, as these pathogens feed on dead tissue (Govrin and Levine, 2000). It is therefore pertinent for plants activate the specific defense responses against invading pathogens. Salicylic acid (SA)-mediated resistance has been seen to be effective against biotrophs, whereas jasmonic acid (JA) or ethylene-mediated responses are predominantly directed against necrotrophs and herbivorous insects (Glazebrook, 2005). Intriguingly, some pathogens are capable of inducing multiple plant signal molecules and hormones, such as SA and JA. In such cases, networking and interplay between these signaling pathways may be the mechanism that allows the plant to prioritize one response over the other. Pathogen infection also has profound implications on hormonal pathways involved in plant growth and development. During the course of evolution many pathogens have come up with mechanisms to tap into these hormone-signaling networks to interfere and suppress host defense. In response, the crosstalk may be used by the host as a direct
defense mechanism against pathogen-triggered manipulation of hormone signaling.

2.3.1 SA-JA cross talk

In recent years insights into the model system *Arabidopsis* has yielded several proteins with an important regulatory roles in SA-JA crosstalk, in relation to signaling on resistance against biotrophs and necrotrophs. Among these molecular players are the mitogen-activated protein kinase, the lipase-like proteins *EDS1* and *PAD4* (*Phytoalexin-Deficient 4*), the defense regulatory protein *NPR1*, the fatty acid desaturase *SSI2*, the Glutaredoxin GRX480 and WRKY transcription factor proteins like WRKY70 (Koomneef and Pieterse, 2008). The majority of the identified crosstalk regulators play pivotal roles in SA signal transduction, in which *NPR1* plays a central role. The *NPR1* acts downstream of *EDS1* and *PAD4* in the SA signaling pathway. In addition, *NPR1* regulates the SA-mediated expression of GRX480 and WRKY70, which encode proteins that suppress JA-dependent gene expression (Ndamukong et al. 2007; Li et al. 2004). However, the *SSI2* mutant was was seen to exert an NPR1-independent role in the regulation of SA-JA crosstalk (Kachroo et al. 2001). Further mutations that restored the lowered fatty acid levels, rescued the *ssi2* mutant phenotype, suggesting a role for fatty acid signaling in SA-JA crosstalk (Kachroo et al. 2001; Kachroo et al. 2003).

In *Arabidopsis*, the ability of SA to suppress JA-responsive genes was shown to coincide with an increase in the level of glutathione, a major determinant of cellular redox homeostasis (Koomneef and Pieterse, 2008). The glutathione biosynthesis inhibitor, 1-buthionine sulfoximine, strongly reduced the suppression of the JA-responsive gene PDF1.2 by SA, suggesting that SA-mediated modulation of the cellular redox state is an important trigger for the attenuation of JA signaling. *NPR1* the key regulator in SA-mediated suppression of JA was shown to attenuate the JA signaling pathway via cytosolic function, in contrast to its nuclear activation of SA-responsive genes (Koomneef and Pieterse, 2008).

2.3.2 JA-ET crosstalk

The interaction between JA and ET signaling pathways is mostly synergistic. The classic example is the regulation of the *Arabidopsis* plant defensin gene *PDF1.2,*
which requires concomitant activation of both the JA and ET response pathways (Penninckx et al. 1998). The ERFs, ERF1 and ORA59, have emerged as principal integrators of the JA and ET signaling pathways (Lorenzo et al. 2003). The expression of both ERF1 and ORA59 is induced by JA and ET and can also be activated synergistically. Additionally, overexpression of ERF1 or ORA59 in the JA-insensitive mutant coil, or ERF1 in the ET-insensitive mutant ein2, constitutively activated the PDF1.2 gene, which indicates that these transcription factors are important nodes of convergence of JA and ET signaling. The basic helix-loop-helix leucine zipper transcription factor MYC2 (formerly called JIN1, for Jasmonate Insensitive1) has been demonstrated to play an important role in the regulation of JA-responsive genes upon induction, by regulating two distinct classes of JA-responsive genes. MYC2 functions as a positive regulator of JA-responsive genes such as VSP2 and LOX2, whereas it acts as a negative regulator of JA/ET-responsive genes such as PDF1.2 that are activated by ERFs (Lorenzo et al. 2004). Thereby the interplay between the ERFs and MYC2 may allow the plant to activate the set of JA-responsive genes that is required for optimal defense against the attacker encountered.

2.3.3 SA-ET crosstalk

ET has emerged as an important modulator of the plant’s defense response to pathogen and insect attack (Von Dahl and Baldwin, 2007). The analysis of tobacco ET-insensitive mutant (Tetr) showed that ET is essential for the onset of SA-dependent SAR that is triggered upon infection by tobacco mosaic virus (Verberne et al. 2003). Moreover, ET was shown to enhance the response of Arabidopsis to SA, resulting in a enhanced expression of the SA-responsive marker gene PR-1 (De Vos et al. 2006). This synergistic effect of ET on SA-induced PR-1 expression was blocked in the ET-insensitive mutant ein2, which indicates that the modulation of the SA pathway by ET is EIN2 dependent and thus functions through the ET signaling pathway. The global expression profiles of P. syringae infected Arabidopsis wild-type and signaling-defective mutant plants provided further evidence of crosstalk between the SA and ET signaling pathways, as many SA-responsive genes were significantly affected in the ein2 mutant background (Glazebrook et al. 2003).
2.3.4. Other signaling molecules networking with SA-JA-ET pathway

Recent studies on *Arabidopsis* have revealed additional defense signaling pathways that contribute to the existing SA, JA and ET response backbone of the induced defense-signaling network. ABA, which was originally discovered as an active modulator of abiotic stresses has becoming increasingly evident in defense signaling pathways as well (Mauch-Mani and Mauch 2005). ABA is connected to the SA-JA-ET network, as it was shown to attenuate JA/ET-dependent gene expression (Anderson et al. 2004) and to affect JA biosynthesis and resistance against JA-inducing necrotrophic pathogens (Adie et al. 2007). Moreover, ABA was demonstrated to antagonize the onset of SA-dependent defenses and SAR (Flors et al. 2008; Yasuda et al. 2008). Intriguingly, NaCl activated abiotic stress had a similar suppressive effect on the SA-dependent SAR in *Arabidopsis*. Conversely, activation of SAR suppressed the expression of ABA-related genes, which indicates that ABA serves as an important regulator that function at the intersection of abiotic and biotic stress responses (Yasuda et al. 2008).

Auxins play a pivotal role in virtually every stage of plant developmental regulation. The auxin response pathway is connected to the SA-JA-ET signaling network in several ways. Auxin has been demonstrated to affect JA biosynthesis (Nagpal et al. 2005); expression of genes involved in JA production (Liu and Wang, 2006) and promote disease susceptibility to *P. syringae*, a process that can be counteracted by SA (Chen et al. 2007). The whole-genome expression profiling of *Arabidopsis* revealed that SA interferes with auxin responses by global repression of auxin-related genes, including the auxin receptor gene TIR1. This inhibitory stimulated effective defenses against the hemibiotrophic pathogens *H. arabidopsidis* and *P. syringae*, resulting in heightened resistance to these pathogens (Wang et al. 2007).

Recently, gibberellins were shown to interact with the SA-JA-ET network as well. Gibberellins are hormones that control plant growth by regulating the degradation of growth-repressing DELLA proteins. These DELLA proteins were found to promote susceptibility to biotrophic pathogens and resistance to necrotrophic pathogens by modulating the relative strength of the SA and JA signaling pathways. Hence, it was postulated that by regulating the stability of DELLA proteins, gibberellins are able to modulate the SA-JA-ET network and affect the final outcome.
Figure 2.5. Crosstalk of phytohormones during plant immune responses
(Adapted from Pieterse et al., 2009)
Review of Literature

Cytokinins often work synergistically with auxins in processes such as cell division and differentiation of plant tissues. They are linked to the response of plants to biotrophic pathogens that alter the host's physiology, such as *Plasmodiophora brassicae*, which causes aberrant root growth in Brassica species. (Siemens et al. 2006). However, not much is currently known about their connection with the SA-JA-ET network.

Brassinosteroids are key regulators in cell expansion and division, differentiation and reproductive development. Brassinosteroids are perceived by the receptor BRI1, which interacts with the receptor-like kinase BAK1 to initiate an intracellular signaling cascade that regulates growth- and development-related processes. Interestingly, BAK1 also interacts with receptors that recognize PAMPs, such as bacterial flagellin, resulting in the initiation of innate immunity (Chinchilla et al. 2007). Pathogen-specific effectors of *P. syringae* have been shown to interfere with this process by binding to BAK1 themselves; they consequently impede the host immune response (Boller and Felix, 2009). However, the role of BAK1 in the innate immune response seems to be independent of the function of BAK1 in brassinosteroid signaling (Kemmerling et al. 2007). Hence, a connection between brassinosteroid signaling and the SA-JA-ET network remains to be established.

2.4. Role of Mitogen-Activated Protein Kinase Cascades in plant signaling

Living cells to adapt to its environment and changes therein, through specific mechanisms that transmits information to the cells and outside it. Such exchange of information helps the cell to adapt to its changing environment by reprogramming its transcriptional and translational machinery. The mechanisms underlying this exchange of information are the processes of protein phosphorylation by specific protein kinases and de-phosphorylation by protein phosphatases (Hardie, 1999). Activation and de-activation of enzymes through phosphorylation/de-phosphorylation by kinases and phosphorylases allows for fast and specific signal transduction, and amplification of external stimuli (Brown et al. 1997). One such signal transduction mechanism, the Mitogen-Activated Protein Kinase (MAPK) cascade, plays an important role in many different eukaryotic organisms, from yeast, to mammals, and
also plants. MAPK were first identified by Sturgill & Ray (1986) from insulin-treated 3T3-L1 cell extracts that would phosphorylate microtubule associated protein-2 (MAP-2) on both serine and threonine residues. MAP-2 kinase was shown to be closely related to a set of previously identified proteins which are tyrosine phosphorylated in response to mitogens, and were renamed p42 MAP kinase (Mitogen-Activated-Protein kinase) (Rossomando et al. 1988).

These protein phosphorylation cascades mediate the intracellular transmission and amplification of extracellular stimuli, resulting in the induction of a plethora of biochemical and physiological cellular responses. MAPKs form the terminal components of these evolutionarily conserved sequential cascades, and are activated by MAPK kinases (MAPKKs or MEKs) via dual phosphorylation of the conserved threonine and tyrosine residues in the motif TxY located in the activation loop (T-loop) between kinase sub-domains VII and VIII. MAPKKs are themselves activated by MAPKK kinases (MAPKKKs or MEKKs) through phosphorylation of conserved serine and/or threonine residues in their T-loop (MAPK G, 2002).

In plants, the MAPK cascades are associated with various physiological, developmental and hormonal responses. Molecular and biochemical studies using specific antibodies to particular MAPKs have revealed that MAPK activation correlates with stimulatory treatments such as pathogen infection, wounding, low temperature, drought, hyper- and hypo-osmolarity, high salinity, touch, and ROS (Fiil et al. 2009). In Arabidopsis genome, 20 MAPK genes have been identified suggesting a high level of complexity in the plant MAPK cascades (MAPK G, 2002). In comparison to mammalian MAPKs, most plant MAPKs share highest homology to the Extracellular Signal-Regulated Kinase (ERK) subfamily. The predicted amino acid sequences of these plant MAPKs show a high degree of conservation over the entire lengths with highest similarity in all the eleven domains necessary for the catalytic function of the serine/threonine protein kinase. However, the N- and C-terminal extensions outside the eleven sub-domains are divergent compared catalytic core and these sequences have important biological function. Comparisons of deduced amino acid sequences indicate that plant MAPKs can be grouped into at least four distinct families (MAPK G, 2002). The Group A MAPKs including Arabidopsis MPK3, MPK4, alfalfa SIMK, tobacco WIPK are mostly involved in environmental and hormonal response. Group B MAPKs are involved in cell cycle regulation and
response to abiotic or environmental stress. The functions of MAPKs in Group C are not well characterized till date. Group D MAPKs, on the other hand, have the TDY motif instead of TEY in their T-loop and also have the extended C-terminal region. Two reported Group D MAPKs, rice BMWK1 and alfalfa TDY1, were found to be induced by blast fungus and wounding respectively (MAPK G, 2002).

Analysis of the Arabidopsis genome for MAPKKs also revealed four different groups with a total of 10 MAPKKs (MAPK G, 2002). The number of MAPKKs in Arabidopsis genome is only half of MAPKs, so MAPKKs are likely to activate multiple MAPKs, and that crosstalk between various signal-transduction pathways might be concentrated at this level in plant MAPK cascades. Plant MAPKKs have the S/T-X5-S/T motif as the phosphorylation site, and a putative MAPK-docking domain K/R-K/R-K/R-X1-6-LX-L/V/I. Several MAPKKs have been identified from different plants, including Arabidopsis MAPKK1 (renamed from MEK1) and M KK2-5, alfalfa SIMKK and PRKK, tomato LeMEK1, tobacco NtMEK1-2 and SIPKK, and maize ZmMEK1. Arabidopsis MKK1 was activated by wounding and abiotic stress (Matsuoka et al. 2002). PRKK was found to activate SIMK, MMK3 and SAMK in response to the fungal elicitor (Cardinale et al. 2002). SIMKK was found to conduct both salt and elicitor induced signals with different substrate specificities (Kiegerl et al. 2002) whereas dexamethasone-induced transient overproduction of a constitutively active form of tobacco NtMEK2, in tobacco, induced not only SIPK and WIPK but also hypersensitive cell death (Yang et al. 2001).

There are about 60 putative MAPKKKs in Arabidopsis and have been divided into two subfamilies, typified by their mammalian homologs: MEKK-like protein kinases, and Raf-like protein kinases (MAPK G, 2002). Group A comprises MAPKKKs whose kinase domains have significant similarity to typical MAPKKKs, such as MEKK/STE11/BCK1. Group A has been further divided into five subgroups. Subgroup A1 comprises four protein kinases (AtMEKK1−4) that have been found to be implicated in enhanced by drought, high salinity and touch (Mizoguchi et al. 1996). AtMAPKKKs classified into Groups B and C are related to the RAF kinases, whose sequences differ from those of MEKK/Ste11/Bck1 (Jouannic et al. 1999). All Group B, RAF-related AtMAPKKKs have extended N-terminal domains. Among them, CTR1 (Kieber et al. 1993) and EDR1 (Frye et al. 2001) are involved in ethylene and disease resistance signaling, respectively. Interestingly, the AtMAPKKKs in
RAF-related subgroup B2 contain Per, Amt and Sim (PAS) domains and PAS-associated C-terminal (PAC) domains in their N-termini; the PAS domain functions as a signal sensory domain in many signaling pathways (Zhulin et al. 1997).

2.4.1. Role of MAP kinases in plant defense signaling

Till date only a few MAPK cascade components have been fully elucidated and their roles studied in detail. The Arabidopsis MAPKKKs YODA, ANP2/ANP3 and MP3K6/MP3K7 have been found to function in development whereas; MEKK1 and ANP1 were found to act in response to environmental stress. In totality, 8 of the 20 MAPKs have been studied to various degrees for the functions they govern (Colcombet and Hirt 2008). The most studied MAPKs are MPK3, MPK4 and MPK6, all of which are activated by a diversity of stimuli including abiotic stresses, pathogens and oxidative stress. While MPK4 negatively regulates biotic stress signalling, MPK3 and MPK6 act as positive mediators of defence responses. The key role of these three MAPKs for normal plant growth and development is evidenced by the severely dwarfed phenotype of mpk4 and the embryo lethal phenotype of mpk3/mpk6 double mutants (Wang et al. 2007b). The flagellin derived peptide flg22 has been documented to trigger a rapid and strong activation of MPK3, MPK4 and MPK6 (Droillard et al. 2008). MPK4 and MPK6 are also activated by harpin proteins, encoded by hrp (hypersensitive response and pathogenicity) genes in many plant pathogenic bacteria. The key role of MPK3/MPK6 in camalexin-based fungal resistance has been demonstrated by the fact that mpk3 and mpk6 mutants are compromised in camalexin production and consequently more susceptible to B. cinerea. Recent data implicate a MAPK cascade composed of MKK4/MKK5 and MPK3/MPK6 in response to fungal pathogens, based on the observation that activation of MPK3/MPK6 in conditional gain-of-function (GOF) plants for MKK4/MKK5 or MEKK1/MKKKa and was sufficient to induce accumulation of camalexin, even in the absence of pathogen attack (Ren et al. 2008). Yet another MAPKK, MKK9, was also found to be involved in camalexin biosynthesis. In addition, the MKK9-MPK3/6 was also found to be involved in the biosynthesis of ethylene, a defense related phytohormone (Xu et al. 2008).
The amplification and transduction of pathogen-derived signals perceived at membrane receptors and further transmittance of these signals into altered gene expression, is the major role played by MAPK cascades in defense signaling. Not all components of MAPK cascades have been thoroughly studied in plants. Hence, the identification and *in planta* verification of additional MAPKKK-MAPKK-MAPK modules and the discovery of downstream offer to help decipher the sophisticated network of plant MAPK signaling pathways and their role in various biological responses.