Chapter 2
Salicylic Acid Signaling in Plant Innate Immunity

Abstract Plants are endowed with innate immune system to protect against invading pathogens. The innate immune system serves as a surveillance system against possible attack by viral, bacterial, fungal, and oomycete pathogens. The innate immune system is a sleeping giant to fight against pathogens, and specific signals are needed to activate them. The pathogen's signature, pathogen-associated molecular pattern (PAMP), switches on the plant innate immune system. The PAMPs are perceived as alarm signals by plant pattern recognition receptors (PRRs), which have a "receptor" and a "signaling domain" in one molecule to perceive and transduce the PAMP signal. Several second messengers are involved in delivering the message generated by the PAMP/PRR signaling complex to plant hormone signals. Salicylic acid (SA) is the important endogenous plant hormone signal in delivering the extracellular PAMP message into the plant cell to initiate the transcription of defense genes. PAMP signaling system generates specific Ca^{2+} signature in the cytosol, which triggers SA biosynthesis. The information encoded in calcium signature is decoded by an array of calmodulins. A calmodulin-binding protein, CBP60g, has been shown to be involved in activating SA biosynthesis. Calcium signature signals transduced to calmodulin-binding protein CBP60g trigger activation of isochorismate synthase in SA biosynthesis pathway. ROS also acts upstream of SA accumulation. H_2O_2 causes an intracellular accumulation of benzoic acid (BA), and the conversion of BA to SA is catalyzed by benzoic acid 2-hydroxylase (BA2H), an inducible enzyme that is synthesized de novo in response to increased BA level. Nitric oxide (NO) activates SA biosynthesis pathway, by inducing phenylalanine ammonia lyase (PAL) which is a key enzyme in biosynthesis of salicylic acid. Several MAP kinase cascades have been shown to act upstream of SA signaling system. SA signaling induces increased expression of transcription factors to activate SA-responsive defense-related genes. NPR1 is a master regulator of the SA-mediated induction of defense genes. NPR1 directly binds SA and binding of SA occurs through $Cys^{521/529}$ via the transition metal copper. Nuclear localization of NPR1 protein is essential for its function. In the absence of pathogen challenge, NPR1 is retained in the cytoplasm. Without induction, NPR1 protein forms an oligomer and is excluded from the nucleus. Pathogen/PAMP exposure induces SA accumulation, and the induced SA controls the nuclear translocation of NPR1

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through cellular redox changes. In the absence of pathogen challenge, NPR1 is continuously cleared from the nucleus by proteasome, which restricts its co-activator activity to prevent untimely activation of defense responses. Two NPR1 paralogues, NPR3 and NPR4, have been identified as adaptor proteins of the CUL3 E3 ligase, and they target NPR1 degradation in an SA concentration-dependent manner. At increased SA concentration after infection, SA binds to NPR4, and NPR1, freed from NPR4 binding, activates transcription of defense genes. NPR1 is a cofactor of TGA transcription factors, and it enhances binding of TGA transcription factors to the promoter of *PR1* gene to activate transcription of *PR1* gene. Systemic acquired resistance (SAR) is a salicylic acid-dependent heightened state of defense against a broad spectrum of pathogens activated throughout a plant following a local infection. Methyl salicylate, methyl salicylate esterase, a lipid transfer protein (DIR1), a lipid-derived molecule (glycerol-3-phosphate)-dependent factor, azelaic acid, dehydroabietinal, and pipecolic acid have been suggested to be the systemic mobile signal molecules involved in SAR. Some Mediators have been shown to be involved in triggering SA-mediated SAR. Mediator is a multiprotein complex that functions as a transcriptional coactivator. SAR is associated with priming of defense, and the priming results in a faster and stronger induction of defense mechanisms after pathogen attack. Some dormant MAPKs have been suggested to be important components required for priming. Pipecolic acid is an endogenous mediator of defense priming. SAR involves extensive reprogramming of transcription. SA mediates changes in the expression pattern of about 1,000–2,000 genes. Such a broad effect on gene transcription may be associated with extensive chromatin remodeling. The chromatin remodeling may involve substitution of canonical histones in the octamer by histone variants, in a process known as histone replacement. Chromatin structure is important for the regulation of gene expression, and chromatin states could control cellular memory. The primed genes may be poised for enhanced activation of gene expression by the histone modification in chromatin. There may be a tight correlation between histone modification patterns and gene priming, and also there may be a histone memory for information storage in the plant stress response. NPR1 may be involved in the chromatin modification-induced priming. NPR1 plays important role in inducing high levels of chromatin modification on promoters of the transcription factor genes. Chromatin remodeling may be instrumental for priming of SA-responsive loci to enable their enhanced reactivation upon subsequent pathogen attack. The priming can be inherited epigenetically from disease-exposed plants, and descendants of primed plants exhibit next-generation systemic acquired resistance. The descendants of primed plants showed a faster and higher accumulation of transcripts of defense-related genes in salicylic acid signaling pathway and enhanced disease resistance upon challenge inoculation with virulent pathogens. The transgenerational SAR was found to be sustained over one stress-free generation, indicating an epigenetic basis of the phenomenon. DNA methylation may also play an important role in transgenerational SAR. The transgenerational SAR is transmitted by hypomethylated genes that direct priming of SA-dependent defenses in the following generations.

2.1 Salicylic Acid as an Endogenous Immune Signal in Plants

Salicylic acid (SA) is an important endogenous immune signal in the induction of disease resistance response in plants (Anand et al. 2008; Fung et al. 2008; Garcion et al. 2008; Mukherjee et al. 2010; Dempsey et al. 2011; Liu et al. 2011a, b; Argueso et al. 2012; Fu et al. 2012; Denancé et al. 2013; Gimenez-Ibanez and Solano 2013; Yang et al. 2013). An increase in endogenous concentration of SA after an infection has been reported in many plant–pathogen interactions, and this increase is correlated to the activation of defense mechanisms (Garcion and Métraux 2006; Iwai et al. 2007; Nobuta et al. 2007; Spoel et al. 2007; Anand et al. 2008; Fabro et al. 2008; Garcion et al. 2008). SA signaling system activates not only local resistance but also systemic acquired resistance (SAR) observed in distal (systemic) tissues. SAR is an SA-dependent heightened defense to a broad spectrum of pathogens that is activated throughout a plant following local infection (Liu et al. 2011a).

Infection of plants by necrotizing pathogens, which induce the accumulation of SA, or treatment of plants with synthetic compounds, which are able to trigger SA signaling, causes the induction of a unique physiological state called "priming" (Slaughter et al. 2012; Po-Wen et al. 2013). SAR is associated with priming of defense (Kohler et al. 2002; Jung et al. 2009; Luna et al. 2012), and the priming results in a faster and stronger induction of defense mechanisms after pathogen attack (Conrath 2011; Po-Wen et al. 2013). The priming can be inherited epigenetically from disease-exposed plants (Pastor et al. 2013a), and descendants of primed plants exhibit next-generation systemic acquired resistance (Slaughter et al. 2012; Luna et al. 2012). The transgenerational SA-induced SAR has also been reported (Luna et al. 2012; Pieterse 2012). These studies show that SA is an important immune signal in plants triggering local, systemic, and also transgenerational systemic disease resistance.

2.2 Biosynthesis of Salicylic Acid in Plants

2.2.1 Phenylalanine Pathway

Different pathways for synthesis of SA have been reported. SA may be synthesized via phenylalanine pathway (Ogawa et al. 2006; Garcion and Métraux 2006; Sawada et al. 2006) or isochorismate pathway (Wildermuth et al. 2001; Garcion et al. 2008). SA is synthesized via both the isochorismate pathway and phenylalanine pathway in *Arabidopsis* (Mauch-Mani and Slusarenko 1996; Wildermuth et al. 2001; Ferrari et al. 2003). However, Ogawa et al. (2006) have shown that SA is synthesized predominantly via the phenylalanine pathway in tobacco.

SA may be synthesized from *trans*-cinnamic acid in the phenylalanine pathway by two different routes. One involves side-chain decarboxylation of cinnamic acid to benzoic acid, followed by 2-hydroxylation to SA (Fig. 2.1). Alternatively,

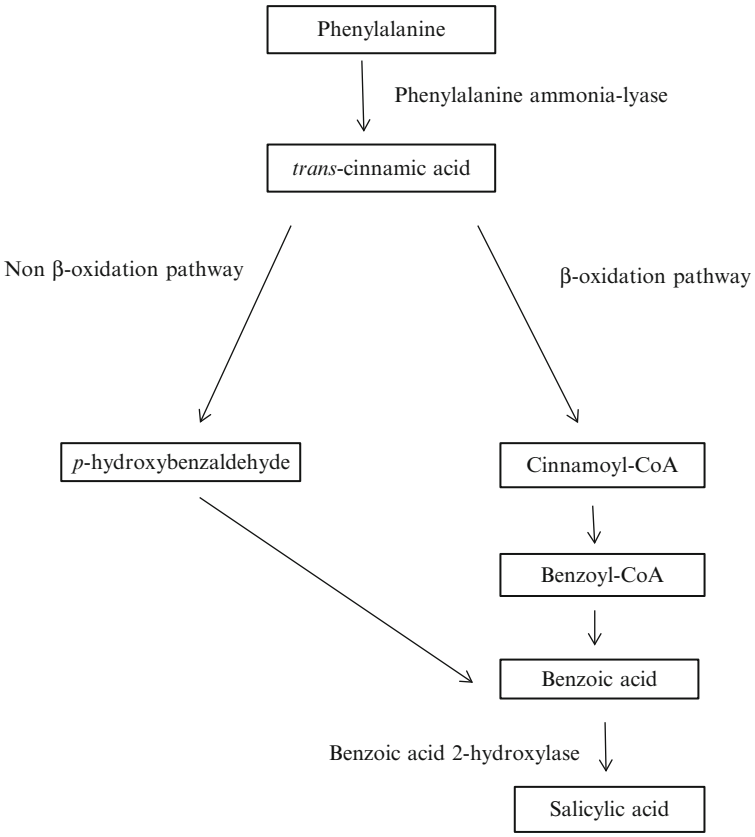

Fig. 2.1 Biosynthesis of salicylic acid in the phenylalanine pathway through benzoic acid route (Adapted from Lee et al. 1995; León et al. 1995; Ribnicky et al. 1998; Chong et al. 2001)

cinnamic acid could be first 2-hydroxylated to *o*-coumaric acid and then decarboxylated to SA (Fig. 2.2) (Lee et al. 1995). *Trans*-cinnamic acid is decarboxylated by two different mechanisms. The first mechanism may operate via β-oxidation, while the other one may be via nonoxidative chain-shortening mechanism (Lee et al. 1995; Fig. 2.1). In the β-oxidation pathway, *trans*-cinnamic acid is converted into cinnamoyl-CoA followed by benzoyl-CoA and then to benzoic acid. In the non-β-oxidation pathway, *p*-hydroxybenzaldehyde is the intermediate in the biosynthesis of benzoic acid (Ribnicky et al. 1998; Chong et al. 2001). Benzoic acid is converted to SA by the enzyme benzoic acid 2-hydroxylase (BA2H). BA2H is a soluble cytochrome P450 oxygenase (León et al. 1993, 1995; Fig. 2.1).

Biosynthesis of SA through *o*-coumaric acid has been demonstrated in tomato seedlings infected with *Agrobacterium tumefaciens* (Chadha and Brown 1974), while biosynthetic pathway via benzoic acid has been reported in tobacco leaves infected with *Tobacco mosaic virus* (Yalpani et al. 1993) and in rice leaves (Silverman et al. 1995).

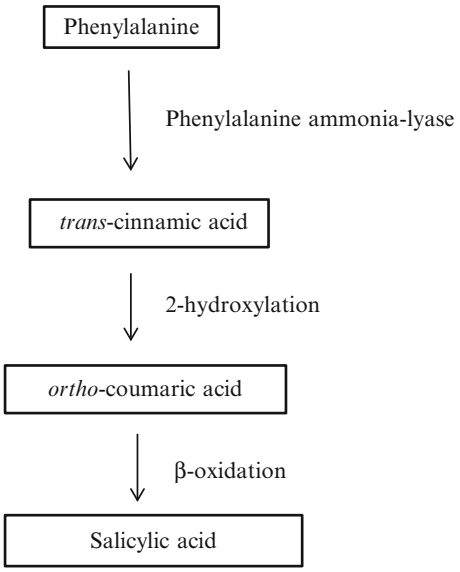

Fig. 2.2 Biosynthesis of salicylic acid in the phenylalanine pathway through *o*-coumaric acid route (Lee et al. 1995)

2.2.2 Isochorismate Pathway

SA synthesis may also proceed through the isochorismate pathway (Wildermuth et al. 2001; Fig. 2.3). In this pathway, SA synthesis proceeds from chorismate via isochorismate, and isochorismate synthase (ICS) is the enzyme involved in the conversion of chorismate to isochorismate (Gaille et al. 2002, 2003). Two ICS genes, *ICS1* and *ICS2*, have been identified in *Arabidopsis* (Garcion et al. 2008). Isochorismate pyruvate lyase (IPL) may be involved in the conversion of isochorismate to SA (Wildermuth et al. 2001; Strawn et al. 2007). However, this enzyme has not yet been reported in plants. No gene encoding proteins similar to bacterial IPL could be detected in *Arabidopsis* (Chen et al. 2009). Two *Arabidopsis* genes, *PBS3* and *EPS*, have been shown to be important in SA biosynthesis. *PBS3* encodes a member of the acyl-adenylate/thioester-forming enzyme family, and *EPS1* encodes a member of the BAHD acyltransferase superfamily. PBS3 (AVR_{ppHB} Susceptible 3) and EPS1 (enhanced pseudomonas susceptibility1) may be the regulatory molecules involved in biosynthesis of SA (Chen et al. 2009; van Verk et al. 2011; Fig. 2.3). The isochorismate pathway has been shown to be active in tomato (Uppalapati et al. 2007) and tobacco (Catinot et al. 2008).

2.2.3 Role of Regulatory Proteins (EDS1, EDS4, PAD4, EDS5, SID2) in Salicylic Acid Biosynthesis

Several regulatory proteins are involved in upstream of SA signaling and involved in SA production in *Arabidopsis* (Fig. 2.4). Mutations in *eds1* (for *enhanced disease susceptibility1*), *eds4*, or *pad4* (for *phytoalexin-deficient4*) lead to reduced SA levels

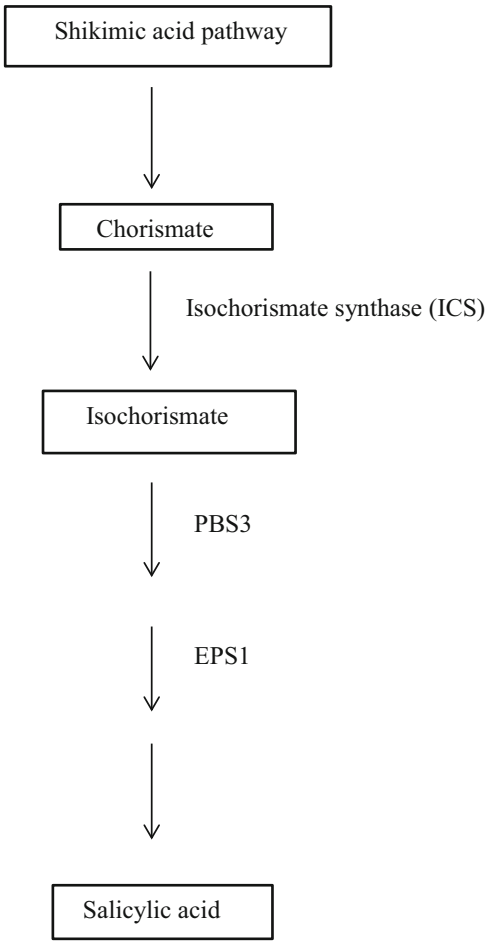

Fig. 2.3 Biosynthesis of salicylic acid in isochorismate pathway (Adapted from Wildermuth et al. 2001; Gaille et al. 2003; Chen et al. 2009)

in infected leaves (Zhou et al. 1998; Gupta et al. 2000; Feys et al. 2001). EDS1 is required for SA production, and it controls SA production to amplify defense signals (Rustérucci et al. 2001; Eulgem et al. 2004). PAD4 is a key regulator acting upstream of SA (Lippok et al. 2007). *Arabidopsis* plants carrying *pad4* mutations have a defect in accumulation of SA upon pathogen infection (Zhou et al. 1998). PAD4 is required for amplification of weak signals to a level sufficient for activation of SA signaling (Jirage et al. 1999). The PAD4 protein sequence displays similarity to triacylglycerol lipases and other esterases (Jirage et al. 1999). EDS1 heterodimerizes with PAD4, and their nuclear localization is important for subsequent steps in the immune signaling pathway (Feys et al. 2001). It is suggested that EDS1 and PAD4 transduce ROS-derived signals leading to SA production (Rustérucci et al. 2001; Mateo et al. 2004; Wiermer et al. 2005). EDS1 and PAD4 may have a fundamental role in transducing redox signals. EDS1 forms several molecularly and

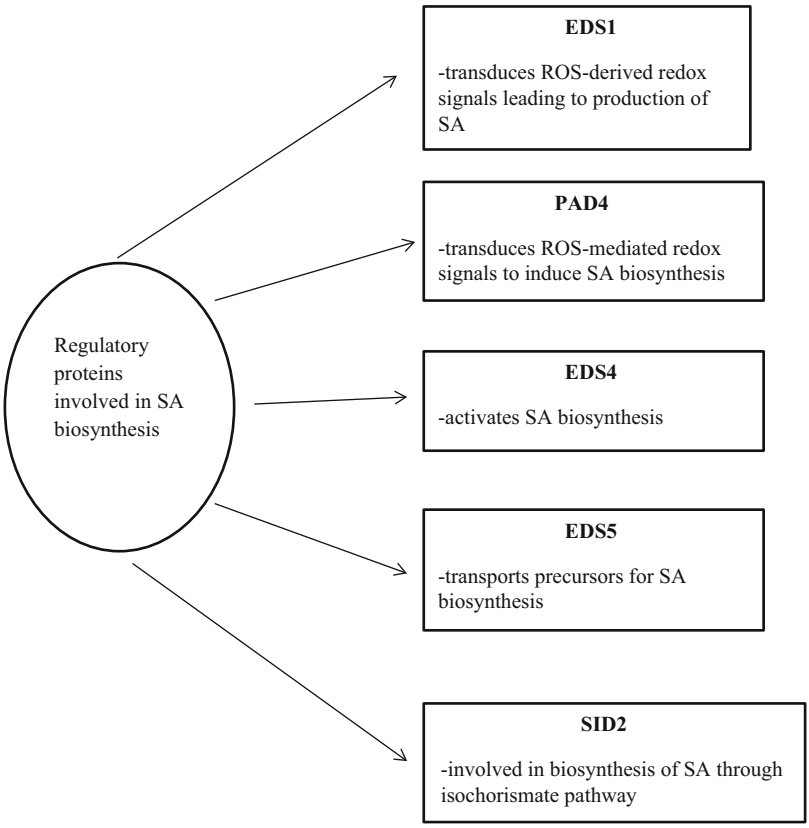

Fig. 2.4 Regulatory proteins involved in salicylic acid biosynthesis

spatially distinct complexes with PAD4 and an in vivo signaling partner, SAG101 (for Senescence-associated gene 101) (Wiermer et al. 2005; Xing and Chen 2006).

SID2 is an isochorismate synthase that is involved in the biosynthesis of SA (Wildermuth et al. 2001). The presence of *sid2* (for *SA induction deficient2*) mutant allele depresses SA synthesis in *Arabidopsis* and compromises the pathogen-induced expression of the *PR1* gene (Wildermuth et al. 2001). The results suggest that SID2 is involved in the biosynthesis of SA through isochorismate pathway. Another gene *EDS5/SID1* encodes a protein, which transports precursors for SA biosynthesis. EDS5 exhibits homology to multidrug and toxin extrusion (MATE) transporter proteins from animals (Nawrath et al. 2002). EDS5 expression requires PAD4, placing EDS5 downstream of PAD4 (Nawrath et al. 2002). EDS4 plays a role in SA signaling and in SA-induced systemic acquired resistance (Gupta et al. 2000). The *eds4* mutant plants showed reduced accumulation of SA induced by *Pseudomonas syringae* pv. *maculicola* infection (Gupta et al. 2000). These results suggest that SA biosynthesis pathway consists of a

linear pathway in which EDS1, PAD4, and EDS4 activate EDS5/SID1and SID2, which produce SA (Glazebrook et al. 2003).

2.2.4 An RNA-Binding Protein (RBP) May Be Involved in SA Biosynthesis Pathway

Gene expression is controlled at both transcriptional and post-transcriptional levels. RNA-binding proteins (RBP) are involved in multiple post-transcriptional processes. After protein-coding genes are transcribed into pre-mRNA by RNA polymerase II, processing and modification steps, such as splicing, are required to produce functional mRNA that is ready for export from the nucleus to the cytoplasm (Lorkovic 2009). The cytoplasmic mRNAs can be translated or degraded. RBP can regulate all of these processes. Plant RBPs are characterized by the presence of RNA-binding domains, such as the RNA recognition motif (RRM) or the K-homology (KH) domain (Lorkovic 2009). Many RBPs have been shown to be involved in plant innate immunity (Fu et al. 2007). An RBP from *A. thaliana*, AtRBP-defense related 1 (AtRBP-DR1), is involved in triggering defense responses against *Pseudomonas syringae* pv. *tomato* DC3000 (Qi et al. 2010). Loss-of-function mutants of *AtRBP-DR1* were found to be more susceptible to the bacterial pathogen than the wild-type plants. Transgenic *Arabidopsis* plants overexpressing *AtRBP-DR1* were developed, and these transgenic plants showed high accumulation of SA (Qi et al. 2010). The loss-of-function *AtRBP-DR1* mutant plants accumulated less SA. AtRBP-DR1 overexpression lines showed higher mRNA levels of *SID2*. The *SID2* gene encodes an isochorismate synthase, which is involved in SA biosynthesis. Activation of the SA pathway by AtRBP-DR1 overexpression was dependent on SID2. The SA-related phenotype in the overexpression line was found to be fully dependent on *SID2* (Qi et al. 2010). Overexpression of *AtRBP-DR1* led to high accumulation of SA, and the plants showed enhanced resistance to *P. syringae* pv. *tomato* DC3000 (Qi et al. 2010). Collectively, these studies showed that the RNA-binding protein DR1 activates the expression of *SID2* gene and enhances isochorismate synthase activity, which triggers the SA biosynthesis (Fig. 2.5; Qi et al. 2010).

2.2.5 GH3.5 Is Involved in Salicylic Acid Biosynthesis

Another gene, *GH3.5* (*Gretchen Hagen3.5*), has been shown to be involved in SA accumulation in *Arabidopsis thaliana* (Zhang et al. 2007b). *GH3.5* is a member of the GH3 family of early auxin-responsive genes in *A. thaliana.* It encodes a protein possessing in vitro adenylation activity on SA. The *A. thaliana* mutant overexpressing GH3.5 showed higher levels of SA compared with wild-type plants inoculated with avirulent strains of *P. syringae*. GH3.5 positively modulates

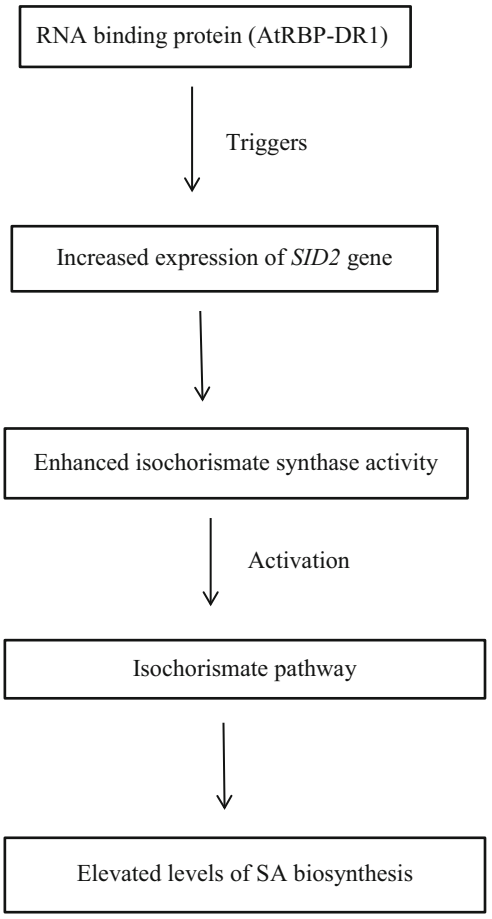

Fig. 2.5 Role of RNA binding protein in triggering salicylic acid biosynthesis (Adapted from Qi et al. 2010)

the SA pathway to enhance plant defense response through elevating SA biosynthesis, activating SA-induced genes, WRKYs, and basal defense-related genes (Zhang et al. 2007b).

2.2.6 *Role of CDR1 Gene in SA Biosynthesis*

An *Arabidopsis* gene, *CDR1* (for *constitutive disease resistance1*), has been shown to take part in reactive oxygen species-mediated SA accumulation in *Arabidopsis* (Xia et al. 2004). It induces accumulation of SA and also induces oxidative burst, which involves H_2O_2 production. The *CDR1* gene encodes an apoplastic protein that shares significant sequence similarity to aspartic proteases. CDR1 contains two active sites with the conserved motifs Asp–Thr–Gly–Ser and

Asp–Ser–Gly–Thr, respectively (Xia et al. 2004). It has been suggested that CDR1 activation might lead to the generation of an endogenous extracellular peptide elicitor. The CDR1-released peptide elicitor might rapidly induce the oxidative burst, and the generated H_2O_2 would have activated SA biosynthesis and accumulation (Xia et al. 2004).

2.2.7 Role of FMO1 Gene in SA Biosynthesis Pathway

Flavin-dependent monooxygenase1 (*FMO1*) gene has been shown to be involved in *EDS1*-mediated SA biosynthesis pathway. *FMO1* positively regulates the *EDS1* pathway (Bartsch et al. 2006). *FMO1* is transcriptionally upregulated in response to superoxide generation but not by hydrogen peroxide (Olszak et al. 2006). An intrinsic activity of EDS1 and PAD4 is to process ROS-derived signals in the defense signaling (Rustérucci et al. 2001; Mateo et al. 2004). FMO may alter the redox state of the EDS1 system (Bartsch et al. 2006) and activate the linear pathway in which EDS1, PAD4, and EDS4 activate EDS5/SID1and SID2, which produce SA (Glazebrook et al. 2003).

2.2.8 Cytokinin May Be Involved in Activation of Salicylic Acid Biosynthesis

Cytokinins are well-known plant growth hormones, and they are also involved in modulation of plant immune responses (Choi et al. 2010, 2011). Cytokinin has been shown to activate SA biosynthesis in *Arabidopsis* (Choi et al. 2010). Within 4 h of the PAMP flg22 application, the expression of *Ics1*, a key SA biosynthetic gene, was hyperactivated 2.7-fold in the presence of the cytokinin t-zeatin (Fig. 2.6; Choi et al. 2010). The increased expression of the *Ics1* gene encoding isochorismate synthase led to elevated SA production (Choi et al. 2010). The results suggest that cytokinin may play an important role in SA biosynthesis pathway.

2.2.9 Some Transcription Factors May Be Involved in Accumulation of Salicylic Acid

Some MYB (*MY*ELO*B*LAST) transcription factors have been shown to play an important role in SA biogenesis. Several *MYB* transcription factor genes are found in plants. They are characterized by the presence of a highly conserved MYB domain at their N-termini (Du et al. 2009). MYB transcription factors contain one or more MYB domains (Stracke et al. 2001). MYB proteins are classified into subfamilies depending on the number of conserved repeats of the MYB domain they

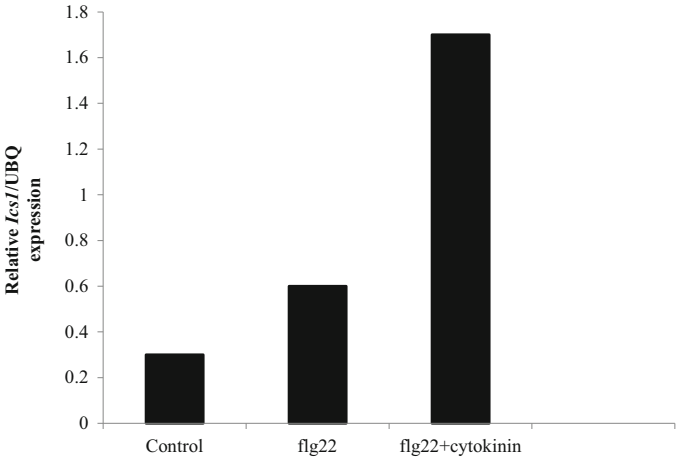

Fig. 2.6 Enhanced expression of the SA biosynthetic gene *ICS1* in cytokinin-treated *Arabidopsis* plants (Adapted from Choi et al. 2010)

contain (Mengiste et al. 2003). A common feature of MYB proteins is the presence of a functional DNA-binding domain that typically consists of one to three repeats of the MYB domain. The three MYB repeats are referred to as R1, R2, and R3. Each repeat is about 50–53 amino acids long and encodes three α-helices, with the second and third helices forming a helix-turn-helix structure which intercalates in the major groove of DNA when bound to it. MYB repeats typically contain regularly spaced tryptophan residues, which build a central tryptophan cluster in the three-dimensional helix-turn-helix fold (Du et al. 2009). MYB transcription factors bind to the *cis-regulatory* element such as MYB-boxes (Laquitaine et al. 2006).

The MYB gene *MYB96* has been shown to be involved in SA biosynthetic pathway (Seo and Park 2010). The *MYB96* gene was rapidly induced by the bacterial pathogen-associated molecular pattern (PAMP) flg22 treatment, and the transcript abundance reached the peak within 1 h. The induction of *SID2* gene, encoding the key SA biosynthesis enzyme isochorismate synthase (ICS), was initiated 2 h after the treatment and reached the peak after flg22 application. The *SID2* gene was significantly up-regulated in the activation-tagging *myb96-1d* line but slightly suppressed in the *myb96-1* mutant (Seo and Park 2010). SA is also synthesized from phenylalanine by phenylalanine ammonia lyase (PAL) activity, although its contribution to endogenous SA content is relatively lower than that of the isochorismate pathway (Lee et al. 1995). The *PAL1* gene was also induced moderately in the activation-tagging *myb96-1d* line (Seo and Park 2010). SA biosynthesis was elevated in the activation-tagging myb96-1d line, and the endogenous concentration of SA was sevenfold higher in the activation-tagging *myb96-1d* line (Fig. 2.7; Seo and Park 2010). These results suggest that PAMP triggers *MYB96* induction, and the induced *MYB96* gene positively regulates the *SID2* gene and also *PAL1* gene to a certain extent, which would result in SA accumulation.

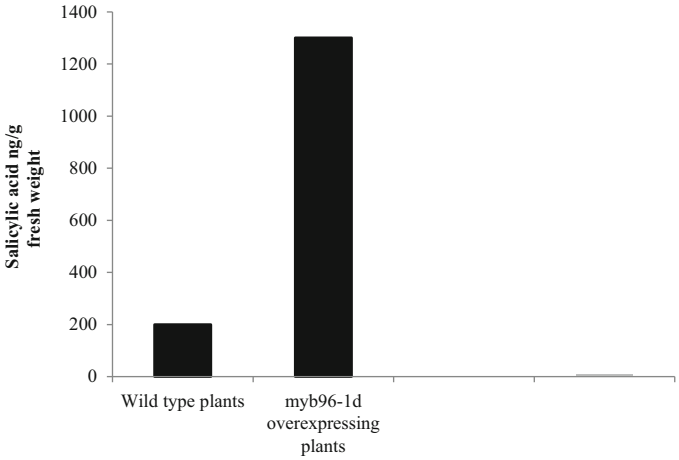

Fig. 2.7 Accumulation of salicylic acid in *Arabidopsis* plants overexpressing *myb96-Id* (Adapted from Seo and Park 2010)

Another transcription factor AtMYB30 has been shown to be involved in SA biosynthesis. AtMYB30 has been shown to be involved in a signaling cascade that modulates (directly or indirectly) SA synthesis. SA operates through the potentiation of reactive oxygen species (ROS) generation, and subsequently, the *ICS* gene encoding isochorismate synthase is positively regulated by AtMYB30, resulting in increased SA production (Raffaele et al. 2006). Alterations of *AtMYB30* expression (overexpression, depletion by antisense strategy, T-DNA insertion mutant) have been shown to modulate SA levels in *Arabidopsis* (Raffaele et al. 2006). *SID1*/*EDS5* and *SID2* which are involved in SA biosynthesis may be activated by AtMYB30 (Raffaele et al. 2006). These studies suggest that AtMYB30 is involved in a signaling cascade that modulates SA synthesis.

Two WRKY transcription factors have been shown to activate SA biosynthetic genes *ICS1* and *PBS3* resulting in accumulation of SA (van Verk et al. 2011). WRKY28 positively regulates the expression of *ICS1* involved in SA biosynthesis pathway. WRKY28 binding sites positioned -445 and -460 base pairs upstream of the transcription start site have been identified in the *ICS1* promoter (van Verk et al. 2011). WRKY46 induces enhanced expression of *PBS3* gene. Overexpression of *WRKY46* resulted in increased accumulation of *PBS3* mRNA, suggesting that WRKY46 is a transcriptional activator of *PBS3* (van Verk et al. 2011). PBS3 (GH3.12) is also involved in pathogen-induced accumulation of SA-O-β-glucoside (SAG), and it regulates SA-dependent defense responses (Nobuta et al. 2007).

Some transcription factors may also act upstream of SA signaling and induce SA biosynthesis pathway. NtWIF is a transcription factor activated upon phosphorylation by wound-induced protein kinase (WIPK) in tobacco plants. The transgenic tobacco

plants overexpressing *NtWIF* exhibited 50-fold higher levels of SA (Waller et al. 2006), suggesting that the transcription factor acts upstream of SA biosynthesis.

2.3 Upstream of Salicylic Acid Signaling System

2.3.1 G-Proteins Trigger Salicylic Acid Biosynthesis in SA Signaling System

Several early signaling events have been shown to be involved in accumulation of SA in plant tissues. G-proteins (Yoda and Sano 2003; Fujiwara et al. 2006), Ca^{2+} signaling network system (Garcia-Brugger et al. 2006), MAPK signaling systems (Andreasson et al. 2005; Waller et al. 2006; Zhang et al. 2007a), ROS signaling system (León et al. 1995; Torres et al. 2006; Ahn et al. 2007), and NO signaling (Durner et al. 1998) act upstream of SA accumulation.

Guanosine triphosphate (GTP)-binding proteins (G-proteins) are the regulatory GTPases (Yalowsky et al. 2010; Zhang et al. 2011). The regulatory GTPases have the ability to bind GTP and hydrolyze it to guanosine diphosphate (GDP). GDP locks G-proteins into their inactive state, while GTP locks G-proteins into their activated state. Active or inactive states of G-proteins depend on the binding of GTP or GDP, respectively (Mucha et al. 2011; Wu et al. 2011). These GTPases act as a simple binary switch (the "off" GDP-bound and the "on" GTP-bound states) (Mucha et al. 2011; Wu et al. 2011). Upon stimulation by an upstream pathogen-associated molecular pattern (PAMP) signal, a G-protein-coupled receptor (GPCR) converts the GDP-bound inactive form of the GTPase into the GTP-bound active form through GDP/GTP replacement (Yang 2002; Oki et al. 2009; Pandey et al. 2010).

G-proteins are involved in plant innate immune signaling system initiated by PAMP signal (Gelli et al. 1997; Park et al. 2000; Zhang et al. 2011). They play an important role in SA biosynthesis. G-proteins may trigger salicylic acid biosynthesis in SA signaling system (Beffa et al. 1995). Cholera toxin from *Vibrio cholerae* is a multimeric protein consisting of A1, A2, and five B subunits. The A1 subunit catalyzes the ADP-ribosylation of heterotrimeric G-protein Gα subunit, and this process irreversibly blocks the GTPase activity of G-proteins leading to the sustained activation of the downstream signaling pathway (Beffa et al. 1995). Cholera toxin does not activate G-proteins directly; it acts to maintain the active state of G-proteins with bound GTP (Beffa et al. 1995). Transgenic tobacco plants expressing A1 subunit of cholera toxin were developed, and tissues of these transgenic plants showed accumulation of high levels of salicylic acid (Beffa et al. 1995). The results suggest that the active form of G-proteins triggers the SA biosynthesis. Sano et al. (1994) reported that expression of a small G-protein in transgenic tobacco abnormally induced salicylic acid in response to an external stimulus. Transgenic tobacco plants expressing a rice gene encoding small GTPase, *rgp1*, showed high accumulation of salicylic acid (Yoda and Sano 2003). These studies reveal that G-proteins are involved in SA biosynthesis.

2.3.2 *Calcium Signaling May Act Upstream of Salicylic Acid Accumulation*

2.3.2.1 Ca^{2+} Signature May Modulate SA Biosynthesis and Accumulation Pathway

The PAMP-activated calcium signaling is modulated by calcium signatures. Ca^{2+} signatures (single calcium transients, oscillations, or waves) are generated in the cytosol and in noncytosolic locations including the nucleus and chloroplast through the coordinated action of Ca^{2+} influx and efflux pathways (Luan et al. 2002; Lecourieux et al. 2006; McAinsh and Pittman 2009). Cytosolic Ca^{2+} signals result from a combined action of Ca^{2+} influx through channels and Ca^{2+} efflux through pumps and cotransporters (McAinsh and Pittman 2009; Ward et al. 2009; Boursiac et al. 2010). Influx occurs down the electrochemical gradient through various ion channels, such as voltage-gated channels or Ca^{2+}-permeable cyclic nucleotide-gated channels (CNGCs) or glutamate-gated ion channels (Qi et al. 2010; Moeder et al. 2011; Vatsa et al. 2011; Price et al. 2012; Vincill et al. 2012). Efflux requires energy-dependent Ca^{2+} pumps (autoinhibited Ca^{2+}-ATPases (ACAs) and ER-type Ca^{2+}-ATPases) (McAinsh and Pittman 2009; Boursiac et al. 2010). The cytoplasmic Ca^{2+} signal is shaped by the balance of activity between Ca^{2+} influx and efflux (Boursiac et al. 2010).

Disruption of the vacuolar calcium ATPases in *Arabidopsis* results in the activation of salicylic acid signaling pathway, probably by generating specific Ca^{2+} signature in the cytosol, which triggers SA biosynthesis (Boursiac et al. 2010). A double knockout mutation of the vacuolar Ca^{2+} pumps ACA1 and ACA11 in *Arabidopsis thaliana* resulted in the activation of SA signaling system triggering programmed cell death. Initiation and spread of hypersensitive response that protects plants from pathogens could also be suppressed by disrupting the production of SA in *Arabidopsis* mutants with combined *aca4/11* mutations and a *sid2* (for *salicylic acid induction-deficient2*) mutation. SID2 is an isochorismate synthase that is involved in the biosynthesis of SA (Wildermuth et al. 2001). These studies suggest that disruption of the vacuolar calcium ATPases may result in the activation of *SID2*-mediated SA biosynthesis pathway (Boursiac et al. 2010).

2.3.2.2 Role of Calmodulin-Binding Proteins in SA Biosynthesis

In plant cells, the calcium ion is a ubiquitous intracellular second messenger carrying the signals generated by perception of PAMPs by pattern recognition receptors (PRRs) of host plants (Kwaaitaal et al. 2011; Nürnberger and Küfner 2011; Ranf et al. 2011; Segonzac and Zipfel 2011; Hamada et al. 2012; Stael et al. 2012). Second messengers are molecules that are used by plants to encode information and deliver it downstream to proteins which decode/interpret signals and initiate cellular responses (e.g., changes in enzyme activity, gene expression) (Snedden and Fromm

2001). Calcium ion acts as a signal carrier (Allen et al. 2000). Calcium signaling is modulated by specific calcium signatures (Lecourieux et al. 2006). The calcium signatures are recognized by different calcium sensors to transduce calcium-mediated signals into downstream events (Wang et al. 2012; Hashimoto et al. 2012).

The Ca^{2+} signature controls diverse cellular processes via Ca^{2+} sensors (DeFalco et al. 2010). Calmodulins are the important Ca^{2+} sensor relays identified in plants (Kang et al. 2006; Takabatake et al. 2007). They function through bimolecular interactions. They undergo a conformational change induced by Ca^{2+} before interacting with and changing the activity or structure of the target protein (Lecourieux et al. 2006). Calmodulins are involved in decoding calcium signals (Lecourieux et al. 2006; Kudla et al. 2010).

The information encoded in calcium signature is decoded by an array of calmodulins (Sanders et al. 2002; Hashimoto et al. 2012). Thus, the extracellular signals are transmitted to calmodulins, and the calmodulin-binding proteins on receiving signals from the calmodulins activate enzymes and trigger transcription of specific genes (Lecourieux et al. 2006; Ma and Berkowitz 2007; Dodd et al. 2010; Reddy et al. 2011). A calmodulin-binding protein, CBP60g, has been shown to be involved in activating SA biosynthesis (Wang et al. 2009). Overexpression of CBP60g in *Arabidopsis* caused elevated SA accumulation, increased expression of the defense genes, and enhanced defense responses and enhanced resistance to *Pseudomonas syringae* (Wan et al. 2012). CBP60g has been shown to participate in SA signaling biosynthesis and accumulation (Wang et al. 2009). It has been suggested that the signal coming from CBP60g may act upstream from SA synthesis, as SA levels are reduced in *cbp60g* mutants (Wang et al. 2009).

The effect of *cbp60g* mutant in SA biosynthesis was most similar to that of *pad4* mutant, suggesting that CBP60 may act upstream of PAD4 (Wang et al. 2009). PAD4, a key regulator of SA signaling system, contributes to SA levels. The *pad4* mutant plants showed reduced accumulation of SA after PAMP treatment (Tsuda et al. 2008). It has been shown that the effect of *cbp60g* mutant in SA biosynthesis was almost similar to that of *sid2* mutant (Wang et al. 2009). It suggests that CBP60g may also act upstream of SID2, an isochorismate synthase that is involved in the biosynthesis of SA (Garcion et al. 2008; Wang et al. 2009, 2011; Truman and Glazebrook 2012). Plants carrying *cbp60g* null mutations were compromised in the induction of *SID2* and accumulation of SA (Wang et al. 2009). The calmodulin-binding protein *CBP60g* has been shown to bind to the promoter region of *SID2* (Zhang et al. 2010). A central domain of CBP60g was found to bind to an oligomer with the sequence GAAATTTTGG selected from the *SID2* promoter (Zhang et al. 2010). CBP60g was shown to bind specifically to a 10-mer oligonucleotide with the sequence GAAATTTTGG (Truman and Glazebrook 2012). CBP60g is strongly induced in response to PAMPs treatment (Wang et al. 2009). PAMP-triggered signaling is greatly affected by the loss of *CBP60g* (Wang et al. 2011). Loss of CBP60g severely impacts the plant's ability to produce SA in response to bacterial inoculation and renders the plant susceptible to infection. These results suggest that the calmodulin-binding protein CBP60g binds with *SID2* gene and promotes SA biosynthesis through activation of SID2 (Fig. 2.8).

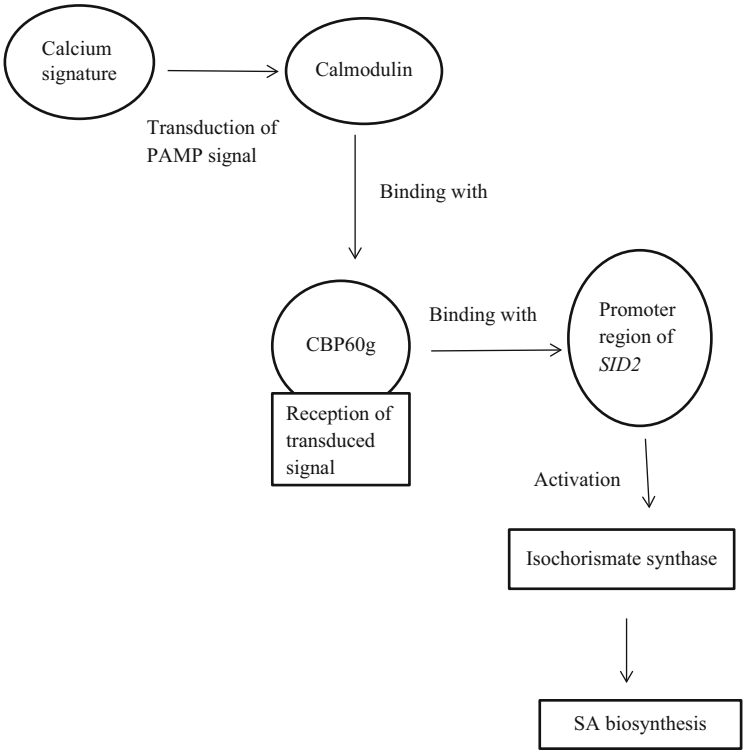

Fig. 2.8 Calcium signature signals transduced to calmodulin-binding protein CBP60g trigger activation of isochorismate synthase in SA biosynthesis pathway (Adapted from Wang et al. 2009, 2011; Truman and Glazebrook 2012; Wan et al. 2012)

One family of CaM-binding proteins, designated as the calmodulin-binding transcription activator (CAMTA) family, resembles a group of putative transcription activators identified in the human genome (Bouché et al. 2002; Galon et al. 2010). This family of proteins contains a transcription activation domain and two types of DNA-binding domains designated the CG1 domain and the transcription factor immunoglobulin domain, ankyrin repeats, and a varying number of IQ CaM-binding motifs (Bouché et al. 2002). *Arabidopsis thaliana* contains six *CAMTA* genes (*AtCAMTA1–AtCAMTA6*). CAMTAs comprise a conserved family of transcription factors (Bouché et al. 2002). The C-terminal CaM-binding domain of CAMTAs mediates interactions with calmodulin (Kudla et al. 2010). CAMTA3 directly interacts with the promoter of the *EDS1* gene, a regulator of salicylic acid levels, and represses its expression (Du et al. 2009). The results suggest that some CaM-binding proteins may negatively regulate SA biosynthesis.

2.3.3 MAP Kinases May Act Upstream of Salicylic Acid Accumulation

PAMPs are perceived as danger signals by PRRs, and the PAMP–PRR complex activates the plant innate immunity. Mitogen-activated protein kinase cascades are major signal transduction systems functioning downstream of PRRs upon perception of PAMP elicitor signals (Hettenhausen et al. 2012; Zhang et al. 2012c). A typical MAPK signaling module consists of three interconnected protein kinases: a MAP kinase kinase kinase (MAPKKK or MEKK [for *M*APK/*E*xtracellular signal-regulated kinase *K*inase *K*inase]), a MAP kinase kinase (MAPKK or MKK), and a MAP kinase (MAPK or MPK) (Mészáros et al. 2006; Li et al. 2012). MAPKs function at the bottom of the three-kinase cascade and are activated by MAPKKs through phosphorylation. The activity of MAPKKs is, in turn, regulated by MAPKKKs via phosphorylation. MAPKKKs receive PAMP signals from upstream receptors/sensors to activate the MAPK signaling system (Hirt 2000; Ichimura et al. 2002; Teige et al. 2004; Li et al. 2012).

Several MAP kinase cascades have been shown to act upstream of SA signaling system. MPK4, its upstream MAP kinase kinases MKK1 and MKK2, and the MAP kinase kinase kinase MEKK1 form a cascade that negatively regulates SA signaling system in *Arabidopsis* (Petersen et al. 2000; Mészáros et al. 2006; Qiu et al. 2008a; Pitzschke et al. 2009). The *mpk4* plants exhibit constitutive systemic acquired resistance, including elevated salicylic acid levels and increased resistance to virulent pathogens (Petersen et al. 2000). The *mekk1/mkk1/mkk2* double mutants also display similar elevated levels of SA (Petersen et al. 2000; Gao et al. 2008; Qiu et al. 2008b). The results suggest that the three-kinase module involving MEKK1–MKK1/2–MPK4 negatively regulates SA biosynthesis and signaling system. Two mitogen-activated protein kinases, salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), are involved in SA signaling system (Ren et al. 2006; Kallenbach et al. 2010; Meldau et al. 2012). WIPK and SIPK function cooperatively to control SA biosynthesis (Seo et al. 2007). Silencing of WIPK/SIPK induced an increase in SA levels (Kobayashi et al. 2010), suggesting that WIPK and SIPK negatively regulate accumulation of SA. EDR1 (ENHANCED DISEASE RESISTANCE 1) is a MAPKK Kinase (MAPKKK), which functions at the top of a MAP kinase cascade. The *edr1* (*enhanced disease resistance 1*) gene encodes a putative MAPKKK, which negatively regulates SA signaling system. All *edr1-associated* phenotypes are suppressed by mutations that reduce SA production (*pad4* and *eds1*) (Frye et al. 2001). The results suggest that EDR1 may negatively regulate PAD4- and EDS1-mediated SA biosynthesis pathway.

Some MAP kinases positively regulate SA signaling pathway in plant innate immunity. Activation of OsMPK6, an ortholog of AtMPK4, resulted in accumulation of SA and enhanced expression of SA signaling genes (Shen et al. 2010). The *MPK7* gene from cotton, *GhMPK7*, has been found to have a role in activating

defense responses in plants. Overexpression of the cotton *MAPK* gene in *Nicotiana benthamiana* induced rapid and strong expression of SA pathway genes (Shi et al. 2010). The MAPKK gene detected in *Arabidopsis*, *MKK7*, positively regulates plant basal and systemic acquired resistance. MKK7 has been shown to trigger accumulation of SA, and the increases in SA levels resulted in enhanced expression of PR genes (Zhang et al. 2007a). The activation-tagged *bud1* mutant, in which the expression of *MKK7* is increased, accumulates SA, exhibits constitutive PR gene expression, and displays enhanced resistance to both the oomycete pathogen *Hyaloperonospora parasitica* and *Pseudomonas syringae* pv. *maculicola* (Zhang et al. 2007a). These results suggest that the MAPKs may act upstream of SA biosynthesis.

2.3.4 *Reactive Oxygen Species May Act Upstream of Salicylic Acid Accumulation*

Hydrogen peroxide (H_2O_2), which is a reactive oxygen species, can also act as a potent signaling molecule (Lehtonen et al. 2012; Petrov and van Breusegem 2012). Pathogen-associated molecular pattern (PAMP) treatment or pathogen infection triggers rapid production of H_2O_2 (Asada 2006; Sagi and Fluhr 2006; Vidhyasekaran 2007; Lehtonen et al. 2012; Petrov and van Breusegem 2012). NADPH oxidases and cell wall peroxidases are the two important groups of enzymes involved in H_2O_2 production (Bolwell et al. 1995, 1998; Suzuki et al. 2011; Daudi et al. 2012; Lehtonen et al. 2012; O'Brein et al. 2012). In rose cells H_2O_2 is produced by a plasma membrane NADPH oxidase, whereas in bean cells H_2O_2 is derived directly from cell wall peroxidases (Bolwell et al. 1998).

H_2O_2 stimulates salicylic acid (SA) biosynthesis in tobacco (León et al. 1995). SA is synthesized in tobacco leaves from benzoic acid (BA) after elicitation (Yalpani et al. 1993). Elicitors trigger the oxidative burst, which results in production of H_2O_2. H_2O_2 causes an intracellular accumulation of BA (León et al. 1993). León et al. (1995) showed that when H_2O_2 was infiltrated into tobacco leaves, the levels of BA increased by more than fivefold. The conversion of BA to SA is catalyzed by benzoic acid 2-hydroxylase (BA2H), an inducible enzyme that is synthesized de novo in response to increased BA level (León et al. 1993). Higher BA levels induce the accumulation of BA2H protein in the cells and provide more substrates for this enzyme. It has been shown that H_2O_2 stimulates BA2H activity (León et al. 1995). BA2H is a soluble Cyt P-450 monooxygenase that uses molecular oxygen for the 2-hydroxylation of benzoic acid (León et al. 1995). In vitro activation of BA2H peroxides was inhibited by a catalase inhibitor 3-amino-1,2,4-triazole (León et al. 1995). The results suggest that the increased 2-hydroxylation activity may be due to the additional oxygen arising from the H_2O_2 degraded by catalase. The catalase-mediated release of molecular oxygen from peroxide may lead to the activation of BA2H, resulting in enhanced accumulation of SA (Fig. 2.9; León et al. 1995).

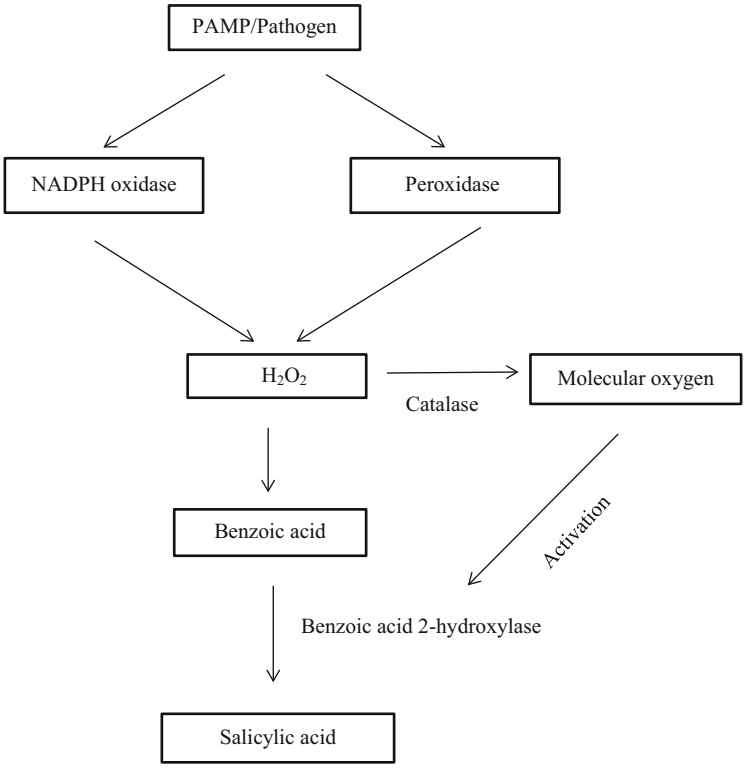

Fig. 2.9 Hydrogen peroxide-stimulated salicylic acid biosynthesis pathway (Adapted from Yalpani et al. 1993; León et al. 1993, 1995; Petrov and van Breusegem 2012)

2.3.5 *Nitric Oxide May Act Upstream of Salicylic Acid Accumulation*

Nitric oxide (NO) is a key mediator for rapid induction of plant immune signaling systems (Neill et al. 2008; Perchepied et al. 2010; Chun et al. 2012; Bellin et al. 2013). An oomycete PAMP/elicitor triggers a NO burst within minutes in tobacco cells (Foissner et al. 2000; Lamotte et al. 2004). A transient burst of NO has been observed in the roots of *Arabidopsis thaliana* as an early response after contact with *Verticillium longisporum* (Tischner et al. 2010). The NO burst has been reported to occur prior to activation of the SA signaling system that eventually activates the transcription of defense genes.

NO has been reported to activate SA biosynthesis pathway. NO is involved in the production of salicylic acid (Durner et al. 1998). NO donors produce SA accumulation (Durner et al. 1998). NO induces phenylalanine ammonia lyase (PAL) which is a key enzyme in the biosynthesis of salicylic acid (Neill et al. 2002). NO triggers

UDP-glucose/SA glucosyltransferase that converts SA to SA β-glucoside, a conjugated and stable form of SA (Zago et al. 2006). NO is capable of inducing expression of the pathogenesis-related protein PR1, which is known to be induced by SA signaling system (Neill et al. 2002). Expression level of SA-inducible *PR1* gene rises following administration of NO donors or expression of recombinant NO synthase in tobacco (Levine et al. 1994). NO is required for the full function of SA as an SAR (systemic acquired resistance) inducer (Song and Goodman 2001).

2.4 Downstream Events in Salicylic Acid Signaling

2.4.1 Generation of Salicylic Acid Conjugates

SA levels in healthy plants are normally low, but rapidly increase upon infection or in induced resistant plants (Malamy et al. 1992; Zhou et al. 1998; Shapiro and Gutsche 2003). Since excess amount of free SA is phytotoxic, diseased/stressed plants regulate free SA levels in part by glucosylation forming SA-O-β-glucoside (SAG) (Fig. 2.10; Enyedi et al. 1992; Malamy et al. 1992; Chong et al. 2001; Shapiro and Gutsche 2003; Song et al. 2008). The conversion of free SA to SAG is catalyzed by UDP-Glc/SA glucosyltransferases (SAGT) (Lim et al. 2002; Song 2006).

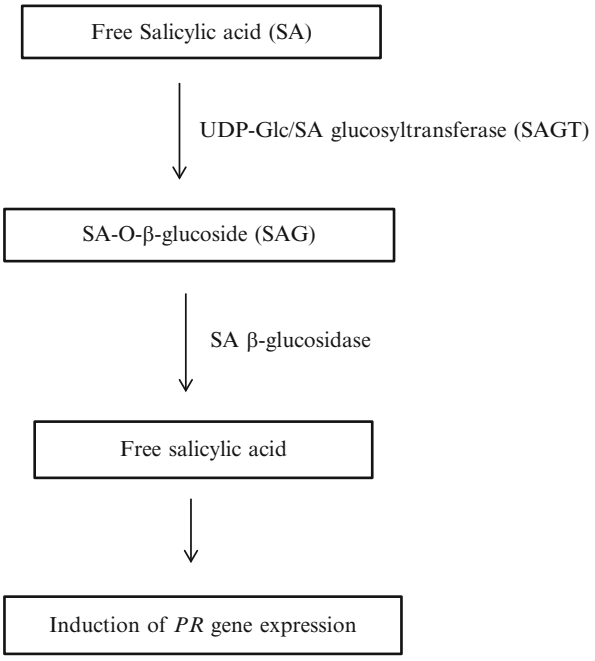

Fig. 2.10 Conversion of free SA to SAG and then to free SA to regulate free SA levels for activation of plant immune responses (Adapted from Seo et al. 1995; Dean et al. 2005; Song et al. 2008)

SA is synthesized in the chloroplast (Strawn et al. 2007), while SAG is formed in the cytoplasm and then transported into the vacuole (Dean et al. 2005). Vacuolar localization of SAG suggests that SAG is primarily a storage form of SA (Nobuta et al. 2007). SAG can be rapidly hydrolyzed to form free SA by endogenous hydrolases (Hennig et al. 1993; Shulaev et al. 1997). SAG is biologically inactive and does not induce *PR* gene expression but can be readily converted back to free active SA by an SA β-glucosidase (Seo et al. 1995).

PBS3 (for *avrPphB* susceptible) (also referred to as GH3.12) is a member of the GH3 protein family of acyl-adenylate/thioester-forming enzymes. GH3 family members act as phytohormone–amino acid synthetases (Staswick and Tiryaki 2004). The *pbs3* mutants are compromised in pathogen-induced accumulation of SAG and expression of *PR1* gene (Nobuta et al. 2007). Exogenous application of SA was sufficient to restore SAG accumulation, *PR1* gene expression, and enhanced disease resistance. The results suggest that the GH3 acyl adenylase is involved in the accumulation of SAG (Nobuta et al. 2007). It is also suggested that GH3 may act directly on SA as an amino acid synthetase to form an SA–amino acid conjugate (Nobuta et al. 2007; Zhang et al. 2007b). The amino acid conjugation may play a critical role in SA metabolism and induced defense responses (Nobuta et al. 2007).

2.4.2 ROS Signaling System May Act Downstream of SA Accumulation

Several effector proteins involved in SA-induced defense responses have been identified. The first protein identified as a salicylic acid-binding protein is the cytosolic (peroxisomal) tobacco catalase (CAT) that reversibly binds SA (Chen et al. 1993; Conrath et al. 1995). SA inhibits CAT's H_2O_2-degrading activity (Durner and Klessig 1996; Wendehenne et al. 1998). SA-mediated inhibition of CAT may generate H_2O_2, which may activate the ROS signaling system inducing expression of defense genes (Chen et al. 1993). Peroxidase has also been reported to interact directly with SA (Durner and Klessig 1995). ROS signaling system may act both upstream and downstream of SA signaling (Slaymaker et al. 2002).

A second specific high-affinity SA-binding protein, SABP2, has been identified in tobacco cytoplasm (Du and Klessig 1997). The third SA-binding protein identified in tobacco is SABP3 (Slaymaker et al. 2002). SABP3 has been detected in the soluble fraction of purified tobacco leaf chloroplasts. SABP3 binds SA with high affinity. Partial sequencing of SABP3 indicated that it is the chloroplast carbonic anhydrase (Slaymaker et al. 2002). SABP3 shows antioxidant activity, and SA may inhibit the antioxidant activity by binding with SABP3. SA's ability to inhibit antioxidant enzymes may play a role in ROS levels, which might activate a positive feedback loop that amplifies SA production and induces defense responses (Slaymaker et al. 2002).

2.4.3 NO May Act Downstream of SA Accumulation

Downstream of SA accumulation, SA has been shown to activate NO synthesis in *Arabidopsis* (Zottini et al. 2007). Many NO-regulated enzymes, including aconitase or catalase, are regulated by SA, suggesting that NO signaling system acts downstream of SA (Durner et al. 1997; Clark et al. 2000). A tight interrelationship between NO and SA in plant defense has been reported (Kumar and Klessig 2000). NO activates salicylate-induced protein kinase (SIPK) in tobacco. Studies with transgenic *NahG* tobacco revealed that SA is required in the NO-mediated induction of SIPK. SIPK may function downstream of SA in the NO signaling pathway (Kumar and Klessig 2000).

NO serves as a key redox-active signal for the activation of various SA downstream defense responses (Klessig et al. 2000). NPR1 and TGA1 are key redox-controlled regulators of SA-induced systemic acquired resistance (SAR) in plants. The translocation of NPR1 into the nucleus has been shown to be promoted by NO (Lindermayr et al. 2010).

NO acts substantially in cellular signal transduction through stimulus-coupled S-nitrosylation of cysteine residues (Benhar et al. 2008). The addition of an NO moiety to a cysteine (Cys) thiol to form an S-nitrosothiol (SNO) is termed S-nitrosylation (Malik et al. 2011). NO reacts rapidly with glutathione (GSH) to yield S-nitrosoglutathione (GSNO) (Espunya et al. 2012). GSNO acts synergistically with SA in SAR (Espunya et al. 2012).

NPR1 is an important regulator of SAR downstream of SA (Mou et al. 2003; Zhang et al. 2003). The events downstream of SA include an increase of NO (Krinke et al. 2007; Zottini et al. 2007). NO is required for the full function of NPR1 in SA-triggered SAR (Song and Goodman 2001). Nuclear localization of NPR1 protein is essential for its function (Kinkema et al. 2000; Meur et al. 2006). Without induction by SA, NPR1 protein forms an oligomer and is excluded from the nucleus. Redox changes cause monomeric NPR1 to emerge and accumulate in the nucleus and activate PR gene expression (Kinkema et al. 2000; Mou et al. 2003). NPR1 is sequestered in the cytoplasm as an oligomer through intermolecular disulfide bonds. NO-mediated S-nitrosylation of NPR1 by S-nitrosoglutathione (GSNO) at Cys156 facilitates the NPR1 oligomerization, which maintains protein homeostasis upon SA induction (Tada et al. 2008). These results suggest that NO is involved in the action of NPR1 in triggering SAR. It also has been shown that NO-induced nitrosoglutathione could act as a long-distance phloematic signal in SA-induced SAR (Durner and Klessig 1999).

2.4.4 MAPK Signaling Cascade May Act Downstream in SA Signaling System

MAP kinase signaling cascade has been widely reported to act upstream in SA biosynthesis pathway (Zhang et al. 2007a; Shen et al. 2010; Shi et al. 2010). It is also known that SA activates MAPK signaling cascade downstream of SA signaling pathway (Zhang et al. 1998; Kumar and Klessig 2000; Zhang and Liu 2001; Cheong

et al. 2003; Uppalapati et al. 2004; Brodersen et al. 2006). SA strongly induced p48 and p44 MAPKs in pea (Uppalapati et al. 2004). It activated a 48-kD MAP kinase and SIPK in tobacco (Zhang and Klessig 1997; Zhang and Liu 2001). A MAPKK encoding gene *GhMKK5* from cotton has been isolated and characterized. *GhMKK5* is significantly induced by SA (Zhang et al. 2012a). The expression of SA signaling system-inducible *PR1a* and *PR5* was greatly elevated in *GhMKK5*-overexpressing *Nicotiana benthamiana* (Zhang et al. 2012a). Another SA signaling pathway gene *NPR1*, which is involved in systemic acquired resistance (SAR) response, was also significantly increased in *GhMKK5*-overexpressing plants (Zhang et al. 2012a). The results suggest that GhMKK5 may act downstream in SA signaling system.

EDR1 (ENHANCED DISEASE RESISTANCE 1) is a MAPKK Kinase (MAPKKK), which functions at the top of a MAP kinase cascade. The *NahG* transgene, which lowers endogenous SA levels, also suppresses *EDR1* expression in *Arabidopsis* (Frye et al. 2001), suggesting that SA triggers expression of *EDR1*. The *EDR1* negatively regulates SA signaling system (Frye et al. 2001).

SA activated the salicylic acid-inducible protein kinase (SIPK) in tobacco (Kumar and Klessig 2000). Studies with transgenic *NahG* tobacco revealed that SA is required for the induction of SIPK and the SIPK may function downstream of SA in the SA signaling pathway (Kumar and Klessig 2000). Another MAPK, MPK4, has been shown to act downstream of SA in the SA signaling system (Petersen et al. 2000). The *mpk4* knockout mutant shows elevated SA levels and constitutively expresses pathogenesis-related (PR) genes (Petersen et al. 2000). Expression of the bacterial NahG salicylate hydroxylase in *mpk4* plants abolishes PR gene expression, indicating the role of the MAPK in SA-mediated signaling system (Petersen et al. 2000; Brodersen et al. 2006). A substrate for MPK4 has been identified and it was designated MKS1 (for *M*AP *K*inase 4 *S*ubstrate *1*) (Andreasson et al. 2005). MPK4 interacts with the nuclear protein MKS1 that in turn interacts with two WRKY transcription factors, WRKY25 and WRKY33 (Andreasson et al. 2005). The molecular phenotypes of plants over- or under-expressing *MKS1* indicate that it mediates some effects of MPK4 on SA-mediated resistance responses. The results suggest that the MKS1 is required for SA-dependent resistance in *Arabidopsis* (Andreasson et al. 2005). The transcription factors WRKY25 and WRKY33 may function as downstream components of the MPK4-mediated signaling pathway and contribute to repression of SA-dependent disease resistance response (Andreasson et al. 2005).

2.5 SA Signaling Induces Increased Expression of Transcription Factors to Activate SA-Responsive Defense-Related Genes

2.5.1 SA Induces WRKY Transcription Factors

Several transcription factors are involved in activation of transcription of defense genes. Proteins containing WRKY zinc-finger motifs constitute a class of transcription factors. WRKY is localized to the nucleus of plant cells and recognizes DNA

molecules containing the TTGAC(C/T) W-box sequence (Zheng et al. 2006). A common feature of all WRKY proteins is the WRKY domain, a highly conserved stretch of about 60 amino acids. Each WRKY domain contains a C-terminal-located novel zinc finger and the strictly conserved amino acid sequence WRKYGQK (tryptophan–arginine–lysine–tyrosine–glycine–glutamine–lysine) at its N terminus. This sequence is required for proper folding of the zinc finger and for DNA binding (Maeo et al. 2001). The WRKY domain binds specifically to various W-box elements, all containing a TGAC core sequence (Wang et al. 1998).

The promoters of a large number of defense-related genes including *PR* genes contain W-box sequences that are recognized by WRKY proteins and are necessary for the inducible expression of these defense genes (Yu et al. 2001). WRKY transcription factors are important regulators of SA-dependent defense responses (Maleck et al. 2000; Wang et al. 2006). In *Arabidopsis*, 74 WRKY transcription factors have been reported (Eulgem et al. 2000), and most of these transcription factors are involved in plant defense responses. Forty-nine of 72 WRKY genes tested were differentially regulated in *Arabidopsis* plants treated with SA (Dong et al. 2003). WRKY proteins have been shown to be strongly involved in alteration of gene expression in response to SA treatment (Eulgem et al. 1999; Dellagi et al. 2000; Asai et al. 2002; Chen and Chen 2002; Yoda et al. 2002; Kalde et al. 2003).

SA induces increased expression of WRKY6, WRKY7, WRKY11, WRKY17, WRKY18, WRKY25, WRKY26, WRKY33, WRKY38, WRKY40, WRKY46, WRKY53, WRKY54, WRKY60, WRKY62, WRKY63, WRKY64, WRKY67, WRKY70, and WRKY75 in *Arabidopsis* (Chen and Chen 2002; Dong et al. 2003; Kalde et al. 2003; Li et al. 2004; Knoth et al. 2007; Mao et al. 2007; Miao and Zentgraf 2007; Zheng et al. 2006, 2007). Expression of WRKY70 is activated by SA, and NPR1 is required for induction of the transcription factor (Li et al. 2004). Overexpression of WRKY70 induced enhanced expression of SA-responsive PR genes (Li et al. 2004). WRKY70 functions downstream of ROS and SA (Knoth et al. 2007). WRKY11 and WRKY17 function as negative regulators of WRKY70 (Journot-Catalino et al. 2006).

Expression of WRKY62 is induced by SA (Mao et al. 2007). SA-inducible WRKY53 transcription factor interacts with a JA-inducible protein, which triggers resistance against bacterial and fungal pathogens in *Arabidopsis* (Miao and Zentgraf 2007). WRKY18 is an SA-inducible transcription factor. It positively modulates defense-related gene expression (Chen and Chen 2002). WRKY18 physically interacts with structurally related *Arabidopsis* WRKY40 and WRKY60, resulting in altered DNA-binding activities (Xu et al. 2006). While constitutive expression of WRKY18 enhanced resistance to *Pseudomonas syringae*, its coexpression with WRKY40 or WRKY60 made plants more susceptible to the pathogen (Xu et al. 2006). SA may induce the activation of WRKY1 through its action on SA-inducible protein kinase (SIPK). SIPK phosphorylates and activates WRKY1, and coexpression of WRKY1 and SIPK results in more rapid induction of defense-related cell

death than overexpression of either alone (Menke et al. 2005). A rice WRKY gene, OsWRKY13, activates both SA synthesis-related genes and SA-responsive genes. It suggests that OsWRKY13 activates genes activating both upstream and downstream of SA (Qiu et al. 2007). A *Vitis vinifera* transcription factor VvWRKY1 is induced by SA. It encodes a polypeptide of 151 amino acids, and it specifically interacts with the W-box in various nucleotide contexts (Marchive et al. 2007). The potato transcription factor StWRKY is also induced by SA (Dellagi et al. 2000).

A novel WRKY transcription factor, NtWRKY12, has been shown to be induced by SA. NtWRKY12 belongs to the class of transcription factors in which the WRKY sequence is followed by a GKK rather than a GQK sequence. The binding sequence of NtWRKY12 (WK-box TTTTCCAC) deviated significantly from the consensus sequence (W-box TTGAC[C/T]) shown to be recognized by WRKY factors with the GQK sequence (van Verk et al. 2008). NtWRKY12 binds with specific binding sites in the *PR1a* promoter in positions -564 (box WK1) and -859 (box WK2). NtWRKY12 and TGA1a act synergistically in *PR1a* expression induced by salicylic acid (van Verk et al. 2008).

Some of the WRKY transcription factors may act as negative regulators of SA-mediated defense gene expression. Some WRKY factors have been shown to negatively regulate pathogen-induced *PR* gene expression (Journot-Catalino et al. 2006; Wang et al. 2006; Xu et al. 2006; Zheng et al. 2007). WRKY48 acts as a negative regulator of SA-induced *PR1* gene expression in *A. thaliana* and basal resistance to *Pseudomonas syringae* (Xing et al. 2008). SA induces WRKY7 gene in *A. thaliana*. However, WRKY7 is a transcriptional repressor in *Arabidopsis*, and transgenic plants overexpressing WRKY7 showed reduced expression of defense-related genes, including *PR1* (Kim et al. 2006). SA induces transcription of WRKY33. However, SA-mediated PR gene expression is retarded by WRKY33, suggesting that WRKY33 negatively acts with SA in inducing defense gene expression (Zheng et al. 2006). WRKY25 is positively regulated by SA signaling pathway. However, its overexpression reduced expression of the SA-regulated PR1 gene. WRKY25 is a substrate of *Arabidopsis* MAP kinase 4, a repressor of SA-dependent defense responses (Zheng et al. 2007).

2.5.2 SA Induces ERF Transcription Factors

SA induces another type of transcription factors, called ethylene response factors (ERF). ERF transcriptional factors are a subfamily of the APETELA2 (AP2) transcription factor family and contain a single DNA-binding domain. The target sequence for ERF transcription factors is the GCC-box that is found in several promoters of PR genes (Grennan 2008). SA induces expression of *TaERF3* in wheat (*Triticum aestivum*) (Zhang et al. 2007b).

2.6 NPR1 Is Master Regulator of SA Signaling

2.6.1 NPR1 Acts Downstream of SA Signal

NPR1 (for *non-expresser of PR gene1*, also known as *NIM1* [*nonimmunity1*] and *SAI1* [*salicylic acid inducible1*]) gene is a master regulator of the SA-mediated induction of defense genes. NPR1 is an important regulator of responses downstream of SA (Mou et al. 2003; Zhang et al. 2003). NPR1 expression levels become elevated upon induction by SA (Cao et al. 1997). *Arabidopsis npr1/nim1* mutants are impaired in their ability to induce PR gene expression even after treatment with SA (Chern et al. 2008), suggesting the function of NPR1 downstream of SA. NPR1 contains a bipartite nuclear localization sequence and two potential protein–protein interaction domains: an ankyrin repeat domain and a BTB/POZ (for Broad complex, Tramtrack, and a Bric-a-brac/Pox virus and Zinc finger) domain (Cao et al. 1997; Ryals et al. 1997). NPR1 functions as a transcriptional co-activator in a TGA2–NPR1 complex after SA treatment. This function requires the BTB/POZ domain and the oxidation of NPR1 Cys521 and Cys529 (Rochon et al. 2006).

NPR1 directly binds SA and binding of SA occurs through $Cys^{521/529}$ via the transition metal copper. NPR1 binds both SA and copper through $^{Cys521/529}$. Removal of metals through chelation abolishes the binding of SA by NPR1, even in the presence of $Cys^{521/529}$. Binding of SA causes a conformational change in NPR1 that is accompanied by the release of the C-terminal transactivation domain from the N-terminal autoinhibitory BTB/POZ domain (Wu et al. 2012).

2.6.2 SA Controls Nuclear Translocation of NPR1

Nuclear localization of NPR1 protein is essential for its function (Kinkema et al. 2000; Meur et al. 2006). In the absence of pathogen challenge, NPR1 is retained in the cytoplasm. Without induction, NPR1 protein forms an oligomer and is excluded from the nucleus. Pathogen/PAMP exposure induces SA accumulation (Durrant and Dong 2004), and the induced SA controls the nuclear translocation of NPR1 through cellular redox changes (Spoel and Dong 2012). Redox changes cause monomeric NPR1 to emerge and accumulate in the nucleus and activate PR gene expression (Kinkema et al. 2000; Mou et al. 2003). NPR1 is sequestered in the cytoplasm as an oligomer through intermolecular disulfide bonds. After induction by SA, the disulfide bonds are reduced, releasing NPR1 monomers into the nucleus. S-Nitrosylation of NPR1 by S-nitrosoglutathione (GSNO) at Cys156 facilitates its oligomerization, which maintains protein homeostasis upon SA induction. Conversely, the SA-induced NPR1 oligomer-to-monomer reaction is catalyzed by thioredoxins (TRX). Mutants in both NPR1 Cys156 and TRX compromised NPR1-mediated disease resistance response. Thus, the regulation of NPR1 is through opposing action of GSNO and TRX. These studies suggest that SA regulates the conversion of NPR1 from an oligomeric to monomeric form, which leads to its nuclear location (Fig. 2.11; Tada et al. 2008).

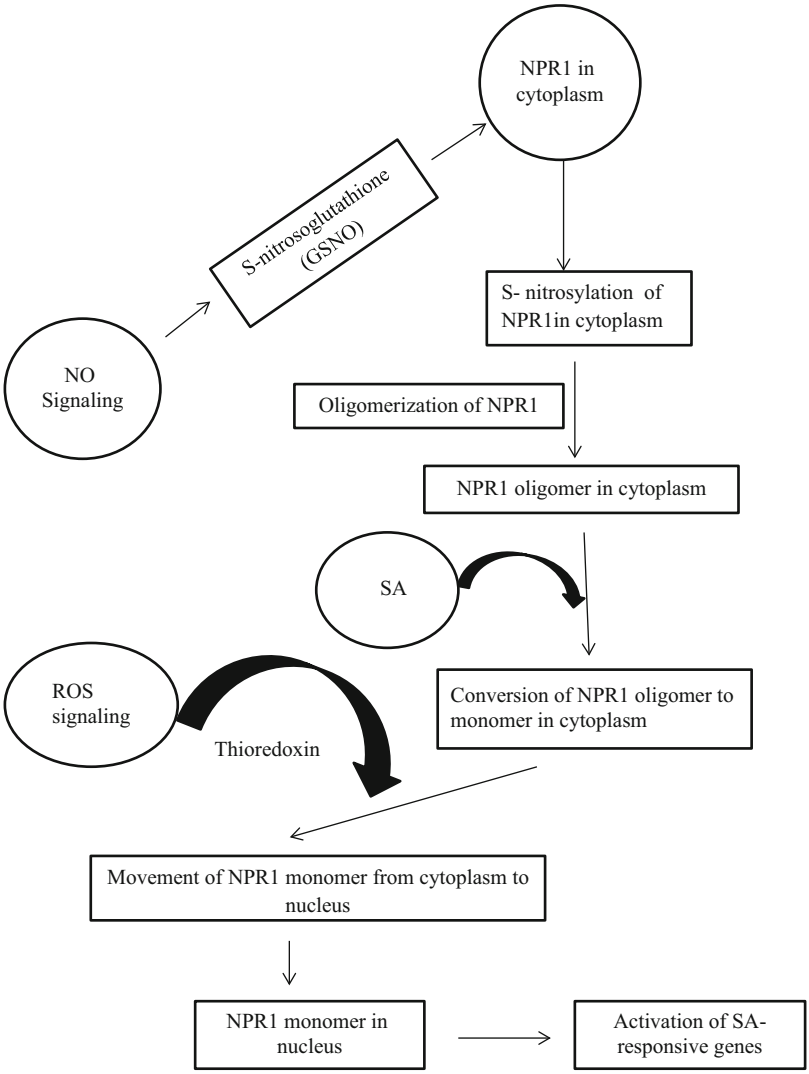

Fig. 2.11 Regulation of NPR1 function through the action of S-nitrosoglutathione and thioredoxin (Adapted from Tada et al. 2008)

2.6.3 SA Modulates Proteasome-Mediated Degradation of NPR1

In the absence of pathogen challenge, NPR1 is continuously cleared from the nucleus by proteasome, which restricts its co-activator activity to prevent untimely activation of defense responses (Spoel et al. 2009). NPR1 protein is degraded by proteasome, and the proteasome-mediated degradation of NPR1 plays an important role in basal disease resistance (Spoel et al. 2009).

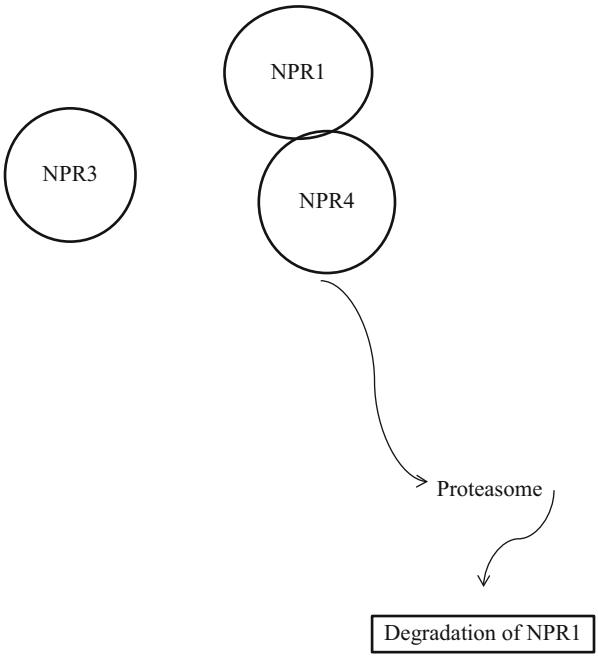

Fig. 2.12 Role of NPR4 in the degradation of NPR1 in plants not exposed to pathogen/PAMP (Adapted from Fu et al. 2012; Moreau et al. 2012)

Proteasomes are large protein complexes located in the nucleus and the cytoplasm (Peters et al. 1994). Proteasomes regulate the concentration of particular proteins. The proteins are tagged for degradation by a small protein called ubiquitin (Pickart and Eddins 2004). Ubiquitin ligase (E3) recognizes the specific protein to be ubiquitinated and catalyzes the transfer of ubiquitin to the target protein (Dreher and Callis 2007). E3 ligases play a key role in the ubiquitin–proteasome system. A Cullin 3 (CUL3) E3 ligase has been found to degrade NPR1 protein (Fu et al. 2012).

Two NPR1 paralogues, NPR3 and NPR4, have been identified as adaptor proteins of the CUL3 E3 ligase. Both NPR3and NPR4 contain the BTP domain as well as an extra protein–protein interaction domain (ankyrin repeat), which are typical for CUL3 substrate adaptors (Fu et al. 2012). The NPR3 and NPR4 adaptor proteins of CUL3 E3 ligase target NPR1 degradation in an SA concentration-dependent manner. At low SA levels found in unchallenged plants, NPR1 is unavailable to induce defense genes, since it is targeted through its binding to NPR4 for degradation in proteasomes (Fig. 2.12; Fu et al. 2012; Moreau et al. 2012). Both NPR3 and NPR4 could bind SA. However, NPR3 had lower affinity in binding SA than NPR4. Accordingly, the binding of SA to NPR3 was slower than NPR4. NPR4 has been shown to have several SA-binding sites (Fu et al. 2012). Hence, it is suggested that SA at low concentrations, NPR1 readily binds with NPR4 and not with NPR3 (Moreau et al. 2012).

At increased SA concentration after infection, SA binds to NPR4 disrupting its interaction with NPR1. NPR1, freed from NPR4 binding, may play its role in activation of defense genes (Fig. 2.13; Moreau et al. 2012).

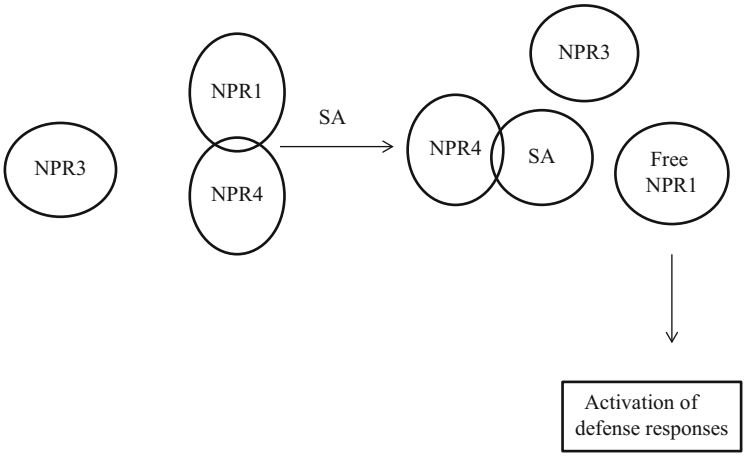

Fig. 2.13 SA binds to NPR4 and frees NPR1 from NPR4 to activate defense gene expression (Adapted from Moreau et al. 2012)

2.6.4 NPR1 Interacting Proteins

Two classes of proteins interact with NPR1/NIM1. The first class includes *Arabidopsis* NIMIN1 (for *NIM In*teracting protein1), NIMIN2, and NIMIN3; tobacco NIMIN2-like proteins (Zwicker et al. 2007); and rice NRR (for *N*egative *R*egulator of disease *R*esistance). Knockout and RNA silencing of *NIMIN1* led to enhanced *PR1* gene expression (Weigel et al. 2005). Constitutive expression of *NtNIMIN2a* led to delayed *PR1* induction, and suppression of *NtNIMIN2a* transcripts enhanced the accumulation of PR1 protein (Zwicker et al. 2007).

A rice gene *NRR* has been identified, and NRR interacts with both the *Arabidopsis* NPR1 protein and the rice NH1 (for rice *N*PR1 *h*omolog*1*) (Chern et al. 2005). Rice NRR protein shows limited similarity to the *Arabidopsis* and tobacco NIMIN2 proteins, only in the NPR1 interaction. NRR behaves similar to NIMIN2 proteins in its interaction with NPR1 (Chern et al. 2005). NRR binds to NPR1 in vivo in a protein complex to inhibit transcriptional activation of *PR* genes, and that NRR contains a transcription repression domain for active repression. Expression of NRR in *Arabidopsis* results in suppression of *PR* gene induction by SA and resistance to pathogens (Chern et al. 2008).

2.6.5 SA-Dependent NPR1-Activated Transcription Factors

The second class of NPR1 interacting proteins belongs to TGA family members of basic-region leucine zipper (bZIP) transcription factors (Zhang et al. 1999; Després et al. 2000; Chern et al. 2001; Fitzgerald et al. 2005). There are 10 TGA transcription

factors in *Arabidopsis* of which seven (TGA1–TGA7) have been characterized with respect to their interaction with NPR1 (Jakoby et al. 2002). TGA2, TGA3, TGA5, TGA6, and TGA7 interact with NPR1 in planta when transiently expressed (Després et al. 2000; Kim and Delaney 2002). The other two TGA factors, TGA1 and TGA4 were found to bind NPR1 only in SA-induced leaves. Reduction of two Cys residues that are uniquely present in TGA1 and TGA4 is responsible for this SA-dependent interaction (Després et al. 2003). Wheat contains several HBP-1b/TGA factors (Mikami et al. 1994). In rice, four NPR1-interacting TGA and TGA-like factors have been identified (Chern et al. 2001). The rice TGA2, TGA2.1, binds with NPR1 (Chern et al. 2001). Rice TGA2.1 binds to oligonucleotides containing the as-1-like (for "activation sequence-1-like") element from the PR1 gene promoter and to the promoter of the rice chitinase gene, RCH10 (Chern et al. 2001).

TGA factors bind to the *as-1* (*cis*-acting) element (Johnson et al. 2003; Rochon et al. 2006). Increased binding to the *as-1* element was observed with SA-induced plant extracts. Binding of in vitro-synthesized TGA2 to the *as-1* element was enhanced in the presence of NPR1 (Després et al. 2000). Depletion of TGA2 and TGA3 from nuclear extracts resulted in reduced protein binding to the *as-1* element (Johnson et al. 2003). TGA2 and TGA3 were recruited to the PR1 promoter in vivo in an NPR1-dependent manner (Johnson et al. 2003). NPR1 may transiently interact with the DNA unbound fraction of TGA2 to promote its recruitment to an active form on cognate target promoters (Johnson et al. 2008). These results suggest that NPR1 enhances the binding of TGA factors to the promoter (Fig. 2.14). TGA-regulated gene expression is very complex. The *PR1* promoter has both positive and negative regulatory *cis*-elements. Mutation in the *LS7* element that contains a TGA-binding site resulted in complete loss of gene induction, whereas mutation in another TGA-binding element (*LS5*) augmented gene expression (Johnson et al. 2003, 2008).

A canonical DNA-binding domain is absent in NPR1. It regulates PR gene expression as a cofactor of the TGA transcription factors, which interact with NPR1

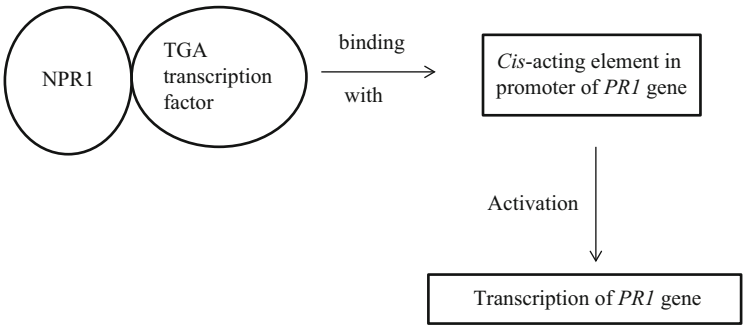

Fig. 2.14 NPR1 as a cofactor of TGA transcription factor enhances binding of TGA transcription factors to the promoter of *PR1* gene to activate transcription of *PR1* gene (Adapted from Fan and Dong 2002; Johnson et al. 2008)

(Fan and Dong 2002). Molecular studies of TGA factors strongly suggest differential effects of TGAs in regulating PR gene expression (Kesarwani et al. 2007). TGA1, TGA3, TGA4, and TGA6 have a positive role in PR gene expression. However, TGA1 and TGA4 have only moderate effects on PR gene expression. TGA2 has repressor activity on PR gene expression, and it acts as a negative regulator of PR genes (Kesarwani et al. 2007). The PR1 promoter contains both positive (LS7) and negative (LS4 and LS5) *cis*-elements. Before induction, TGA2 represses the *PR* genes through interaction with the negative elements. TGA1, TGA3, and TGA6 may bind to the positive element in the uninduced state. This does not lead to *PR* gene expression in the presence of TGA2. It is suggested that upon induction, TGA2 repression is removed and transcription is activated by NPR1 in association with the positive TGA transcription factors, including TGA1, TGA3, TGA5, and TGA6 (Kesarwani et al. 2007).

2.6.6 SA-Induced Expression of PR Genes, Independent of NPR1

In some cases, SA may trigger the induction of *PR* genes, independent of NPR1 (Desveaux et al. 2004). *PR1* is positively regulated in an SA-dependent, but NPR1 independent, manner by the "Whirly" transcription factor, WHY1 in *Arabidopsis* (Desveaux et al. 2004). In rice, *WRKY45* acts in the SA signaling pathway independently of rice NPR1 (NH1) (Shimono et al. 2007). Two defense-related genes, encoding a glutathione *S*-transferase and a cytochrome P450, were found to be regulated downstream of *WRKY45* but were not regulated by the rice NPR1 (NH1). The results suggest the apparent independence of WRKY45- and NH1-dependent pathways. In *Arabidopsis*, most SA-responsive genes are regulated downstream of NPR1, and this regulation is mediated by several WRKY transcription factors acting downstream of NPR1. In rice, WRKY45 and NH1 constitute apparently independent signaling pathways. GST and cytochrome P450 genes are regulated by WRKY45 but not by NH1. *PR1a* and *PR1b* are dependent on NH1 and are conditionally dependent also on WRKY45. Post-translational signal flow from WRKY45 would account for the dependence of these genes on both NH1 and WRKY45. The *lipoxygenase* gene does not appear to depend on either NH1 or WRKY45, so it may be regulated by another regulator (Shimono et al. 2007).

2.7 Role of SUMO in SA Signaling System

The SA-induced changes in gene expression have been found to have a link to chromatin remodeling, such as histone modifications and histone replacement. The recruitment of chromatin-modifying complexes to SA-responsive loci controls their basal and SA-induced expression (March-Diaz et al. 2008; van den Burg and Takken

2009, 2010; Jaskiewicz et al. 2011). Basal repression of these loci may require the post-translational modifier SUMO (for SMALL UBIQUITIN-LIKE MODIFIER). SUMO conjugation has been reported to control the activity, assembly, and disassembly of chromatin-modifying complexes to transcription complexes (van den Burg and Takken 2009). SUMO conjugation determines recruitment and activity of chromatin-modifying enzymes and thereby indirectly controls SA-induced gene expression (van den Burg and Takken 2010).

The *SIZ1* gene, which encodes an *Arabidopsis* SUMO E3 ligase, regulates innate immunity. Mutant *siz1* plants exhibit constitutive SAR characterized by elevated accumulation of salicylic acid and increased resistance to *Pseudomonas syringae* pv. *tomato*. Transfer of the *NahG* gene to *siz1* plants results in reversal of these phenotypes back to wild type. Analyses of the double mutants *npr1 siz1*, *pad4 siz1*, *ndr1 siz1* revealed that *SIZ1* controls SA signaling (Lee et al. 2007).

2.8 SA Induces Transcription of Various Defense Genes

SA triggers transcription of a multitude of genes in plants. More than 790 genes have been shown to be regulated by SA in *Arabidopsis* (Krinke et al. 2007), and most of them are defense response genes. SA induces *PR1* gene expression in *A. thaliana* (Takahashi et al. 2004; Edgar et al. 2006; Kim et al. 2006; Xing et al. 2008). It induced expression of *BGL* (Edgar et al. 2006) and *BG3* (Stein et al. 2006) genes encoding β-1,3-glucanase, *PR4* gene (Stein et al. 2006), and *PR5* gene (Takahashi et al. 2004; Stein et al. 2006) in *A. thaliana*. SA also triggers the activation of various genes encoding different chitinases in *Arabidopsis* (Stein et al. 2006).

SA induces *PR1a* (van Verk et al. 2008), *PR1b* (Nie 2006), and *PR2* genes expression in tobacco (Waller et al. 2006). The expression of acidic *PR1*, *PR2*, and *PR3* genes was up-regulated by SA treatment in tobacco (Niki et al. 1998). SA induces *PR1* expression in potato (Navarre and Mayo 2004). SA induces *OsPR1b* in rice and its expression is downstream of OsWRKY71 (Liu et al. 2007). Acidic *PR1* and *PR2* promoters are induced via SA-dependent signaling pathway in tomato (Hondo et al. 2007).

SA treatment induced peroxidase activity and suppressed replication/accumulation of *Potato virus Y* in tobacco (Nie 2006). SA induced callose deposition in *Arabidopsis* (DebRoy et al. 2004). It also induced transcription of the disease resistance genes *RPW8.1* and *RPW8.2* in *Arabidopsis* (Xiao et al. 2003).

2.9 Role of SA Signaling in Stomatal Closure-Related Immune Responses Against Bacterial Pathogens

SA may trigger defense responses by preventing bacterial entry into host tissue. Bacterial entry into host tissue is a critical first step in causing infection by bacterial pathogens in plants (Melotto et al. 2006; Gudesblat et al. 2009). Stomata serve as

passive ports of bacterial entry during infection. The PAMP-activated innate immune response in *Arabidopsis* involves stomatal closure, which occurs within the first hour of contact with plant tissue (Melotto et al. 2006). The stomatal defense against bacterial pathogens observed in *Arabidopsis* was shown to be under the control of the defense signaling molecule SA. The regulation of stomatal defense by SA suggests that stomatal defense is an integrated part of the SA-regulated immune system (Melotto et al. 2006). The bacterial pathogen *Pseudomonas syringae* pv. *tomato* reopens the closed stomata and counteracts the PAMP-induced/SA-mediated stomatal closure, by producing virulence factors, specifically the phytotoxin coronatine. The toxin overcomes or suppresses SA-dependent defenses (Brooks et al. 2005) and probably suppresses the stomatal defense.

2.10 SA Induces Resistance Against Viruses by Modulating AOX-Mediated Alternative Respiratory Pathway

SA is an important component in the signal transduction pathway leading to systemic resistance that is effective against a broad spectrum of oomycete, fungal, bacterial, and viral pathogens. The hallmark of SA-mediated disease resistance system is the induction of pathogenesis-related (PR) proteins through the function of *NPR1* gene which acts downstream of SA production. PR proteins show antifungal, antioomycete, and antibacterial activity (Vidhyasekaran 2007), but so far no antiviral activity of PR proteins has been reported. It is suggested that SA signaling pathway inducing resistance against viruses may be different from the known resistance pathways inducing resistance against bacterial and fungal pathogens (Ji and Ding 2001).

Mitochondrial signaling processes regulate some aspects of SA-induced virus resistance (Hammerschmidt 2009; Carr et al. 2010). Reactive oxygen species (ROS) are constantly generated within mitochondria as by-products of respiratory electron transport chain activity. Perturbation in this ROS pool or in mitochondrial redox can function in the intracellular signal transduction (Maxwell et al. 1999). This form of signaling is influenced by alternative oxidase (AOX). AOX is a mitochondrial enzyme that is the sole component of the alternative respiratory pathway (Fu et al. 2010; Lee et al. 2011b).

Salicylhydroxamic acid (SHAM), an inhibitor of the mitochondrial enzyme AOX, antagonized the inhibitory effect of SA to viruses but not to fungal or bacterial pathogens (Chivasa et al. 1997). SA-mediated resistance to *Cucumber mosaic virus* (CMV) is attributed to the inhibition of systemic virus movement, which also is sensitive to SHAM (Naylor et al. 1998). Both inhibition and enhancement of AOX have been shown to inhibit the induction of virus resistance, probably by disruption of redox signaling in the mitochondrion (Gilliland et al. 2003; Murphy et al. 2004).

SA-induced resistance against viruses uses signal transduction pathways different from those regulating PR gene expression, constituting a different branch of the SA activity program, in which expression of AOX-1 is involved (Wong et al. 2002).

SA-mediated resistance against viruses follows a distinct branch of SA signaling pathway in tobacco, which is independent of PR gene expression, sensitive to SHAM, and associated with *Aox* induction (Chivasa et al. 1997; Murphy et al. 1999). An early and transient induction of *AOX-1* was observed in response to *Plum pox virus* (PPV) in wild-type plants (Alamillo et al. 2006). Induced resistance against *Turnip crinkle virus* also is SA dependent, yet NPR1, ethylene, and JA independent (Kachroo et al. 2000).

2.11 SA Triggers Small RNA-Directed RNA Silencing System

Small RNA-directed RNA silencing is a potent immune surveillance system targeting foreign nucleic acids of invading pathogens (Ding and Voinnet 2007; Jaubert et al. 2011). The RNA silencing pathway in plants presents a formidable defense against viral pathogens (Qu and Morris 2005). Double-stranded RNA (dsRNA) is the starting point of the antiviral RNA silencing system. RNA silencing is triggered by dsRNA which is commonly generated during plant virus replication (Willmann et al. 2011). In case of single-stranded RNA (ssRNA) viruses, the viral RNA-dependent RNA polymerase (RdRP) encoded by the plant copies a plus-sense ssRNA generating a dsRNA molecule (Qi et al. 2009; Garcia-Ruiz et al. 2010; Wang et al. 2010). In case of other RNA viruses, the two strands do not anneal but can fold into highly structured molecules that have dsRNA regions (Alvarado and Scholthof 2009). In case of geminiviruses, the RNAs transcribed from their circular genomes act as a source of dsRNA (Chellappan et al. 2005). Viroids form hairpin structures, which contain intervals of dsRNA (Papaefthimiou et al. 2001). The nucleic acids of the viral pathogens may be the pathogen-associated molecular patterns (PAMPs), and Ding (2010) suggested that the viral double-stranded RNA (vdsRNA) is the viral PAMP.

The plant innate immune system (defense surveillance system) detects the presence of dsRNA as aberrant RNA molecule (Wypijewski et al. 2009) and generates small RNAs. The generated small RNAs direct the antiviral machinery to cleave and destroy the invading viral genome (Alvarado and Scholthof 2009). In the RNA silencing pathway, small interfering RNAs (siRNAs) are derived from perfectly paired double-stranded RNA (dsRNA) precursors. These dsRNA precursors are derived by the action of RNA-dependent RNA polymerase (RDR or RdRP) (Katiyar-Agarwal and Jin 2010). Primary small RNAs are generated from dsRNA precursors by the action of DCL enzymes. These small RNAs can serve as primers for host RNA-dependent RNA polymerases to generate additional dsRNA targets for DCL enzymes to amplify the silencing signal (Voinnet 2008; Vaistij and Jones 2009; Garcia-Ruiz et al. 2010). The silencing signal is amplified by the action of RNA-dependent RNA polymerase (RDR/RdRP) (Voinnet 2008). The increased expression of RDR1 increases the resistance based on RNA silencing against viruses (Leibman et al. 2011).

Tobacco lines deficient in the inducible RdRP activity were obtained by expressing antisense RNA for the *NtRDRP1* gene in transgenic plants. When infected by *Tobacco mosaic virus* (TMV), these transgenic plants accumulated significantly higher levels of viral RNA and developed more severe disease symptoms than wild-type tobacco plants. After infection by a strain of *Potato virus X* that does not spread in wild-type tobacco plants, the transgenic *NtRDRP1* antisense plants accumulated virus and developed symptoms not only locally in inoculated leaves but also systemically in upper uninoculated leaves (Xie et al. 2001). These results suggest that RdRP plays an important role in plant antiviral immunity.

Several studies have indicated that the RDRs (RdRPs) are inducible by SA (Ji and Ding 2001; Gilliland et al. 2003; Yu et al. 2003; Jovel et al. 2011, Lee et al. 2011b). The activity of a tobacco RdRP was increased in SA-treated plants. Biologically active SA analogs capable of activating plant defense also induced the RdRP activity, whereas biologically inactive analogs did not (Xie et al. 2003). A tobacco *RdRP* gene, *NtRDRP1*, was isolated and found to be induced by treatment with SA or its biologically active analogs (Xie et al. 2003). *Arabidopsis* RNA-dependent RNA polymerase gene (*AtRdRP1*) encoding RdRP1 is induced by salicylic acid treatment in *Arabidopsis* (Yu et al. 2003).

Transgenic rice plants expressing the *Arabidopsis NPR1* (*AtNPR1*) gene showed a higher susceptibility to infection by the *Rice yellow mottle virus* (RYMV), which correlated with a mis-regulation of RYMV-responsive expression of the SA-regulated RNA-dependent RNA polymerase 1 gene (*RDR1*) (Quilis et al. 2008). RDR1-dependent viral siRNAs confer SA-dependent resistance against *Cucumber mosaic virus* by directing non-cell-autonomous antiviral silencing (Diaz-Pendon et al. 2007). Application of exogenous SA enhances virus resistance possibly by amplifying the activity of RDR1 (Ji and Ding 2001; Yu et al. 2003; Jovel et al. 2011). The RdRP gene from cotton, *GhRdRP*, is induced by salicylic acid (SA) and 5-chloro salicylic acid (5-CSA) (Gao et al. 2009).

The viral suppressor of RNA silencing (VSR) 2b protein detected in CMV suppressed non-cell-autonomous transgene silencing and SA-mediated virus resistance. The 2b expressed from the CMV genome drastically reduced the accumulation of 21-, 22-, and 24-nt classes of viral siRNAs produced by DCL4, DCL2, and DCL3, respectively. Disease symptoms caused by CMV-Δ2b in *Arabidopsis* mutants defective in antiviral silencing were as severe as those caused by CMV. The results suggest that the VSR 2b may have an indirect role in virus virulence. Production of CMV siRNAs without 2b interference depended largely on RDR1 inducible by SA. It has been suggested that 2b inhibits the production of RDR1-dependent viral siRNAs that confer SA-dependent virus resistance by directing non-cell-autonomous antiviral silencing (Diaz-Pendon et al. 2007). Collectively, these results suggest that SA signaling may enhance the efficiency of RNA silencing pathway in triggering immune responses against viruses by activating RdRP/RDR.

2.12 Enhancement of Small RNA-Directed RNA Silencing by Salicylate Signaling System

Both SA signaling system (Lee et al. 2011b) and RNA silencing system (Leibman et al. 2011) have been shown to be involved in plant immunity against viral pathogens. When both the systems are expressed simultaneously, resistance responses against the virus diseases appear to be enhanced (Jovel et al. 2011). *Tomato ringspot virus* (ToRSV, a *Nepovirus* sp.) systemically infects many herbaceous plants. In these host plants, viral RNA accumulates in symptomatic leaves and in young, asymptomatic leaves that emerge late in infection, suggesting systemic spread of this virus in host plants. However, systemic infection by the virus was restricted in tobacco, and viral RNA did not accumulate in asymptomatic young emerging leaves. ToRSV-derived siRNAs and SA signaling-derived *PR1a* transcripts were detected only in tissues that contained viral RNA, indicating local induction of RNA silencing and SA-dependent defense responses. Lesion size and viral systemic spread were reduced with SA pretreatment but enhanced in *NahG* transgenic plants deficient in SA accumulation (Jovel et al. 2011). It suggests that SA treatment enhances local defense responses and also it is exclusively involved in suppression of systemic infection by the virus in tobacco. Knocking down the SA-inducible RNA-dependent RDR1 exacerbated the necrotic reaction but did not affect viral systemic spread. The results suggest that SA signaling and not RNA silencing is involved in restriction of systemic infection (Jovel et al. 2011).

Plum pox virus (PPV) is able to replicate in inoculated leaves of *Nicotiana tabacum*, but is defective in systemic movement in this host. PPV produced a systemic infection in transgenic tobacco expressing the P1/HC-Pro, a viral suppressor of RNA silencing (VSR). The results indicate the involvement of RNA silencing in defense against systemic infection of PPV in *N. tabacum* (Alamillo et al. 2006). PPV was able to move to upper noninoculated leaves of tobacco plants expressing bacterial hydroxylase (NahG) that degrades SA. Replication and accumulation of PPV were higher in the locally infected leaves of plants deficient in SA or expressing the VSR. Accumulation of virus-derived small RNA was reduced in the NahG transgenic plants. Systemic infection of PPV was highly enhanced in NahG×P1/HC-Pro double-transgenic plants. Collectively, these results suggest that SA signaling system might act as an enhancer of the function of RNA silencing system in tobacco (Alamillo et al. 2006).

2.13 Interplay Between SA-Induced AOX-Mediated Redox Signaling and SA-Induced Small RNA-Directed RNA Silencing

The gene encoding RNA-dependent RNA polymerase 1 (RDR1) is involved in RNA silencing-mediated basal resistance to several viruses (Rakshandehroo et al. 2009; Leibman et al. 2011). Reducing the accumulation of tobacco *RDR1* (*NtRDR1*)

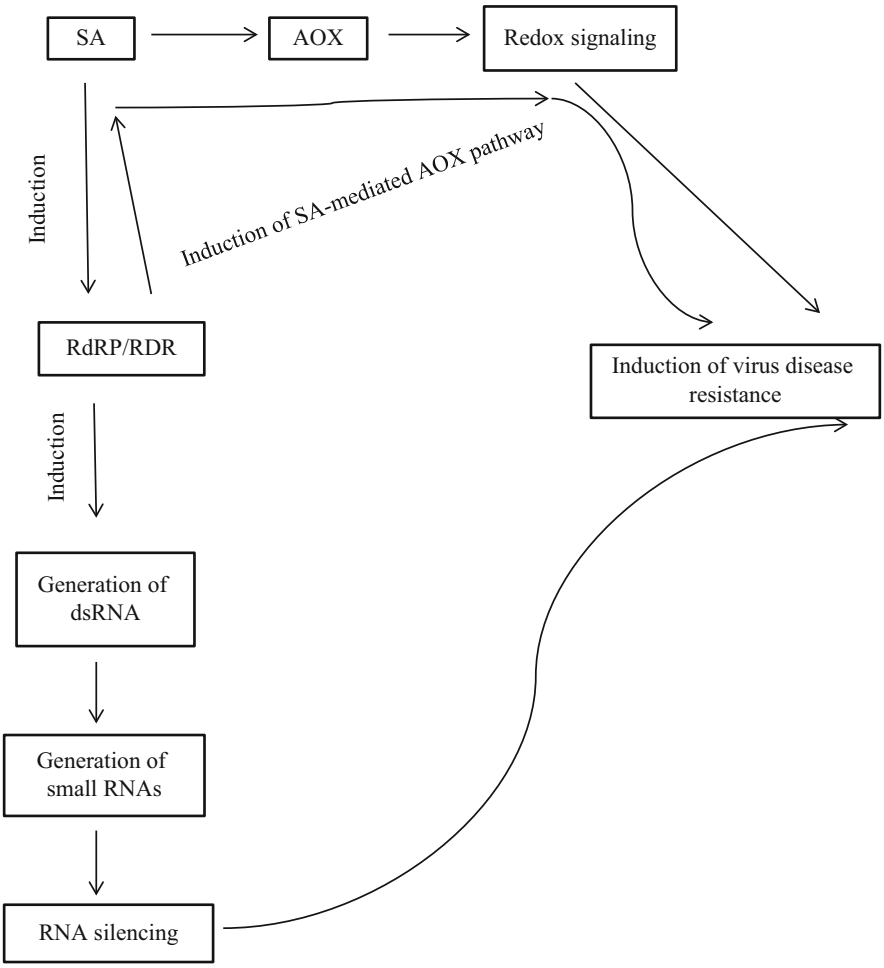

Fig. 2.15 Interplay of SA-triggered AOX-mediated ROS signaling and RNA silencing pathways in inducing virus disease resistance (Adapted from Ji and Ding 2001; Rakshandehroo et al. 2009; Jovel et al. 2011)

transcript by RNA inhibition mediated by transgenic expression of a double-stranded RNA hairpin corresponding to part of *RDR1* gene resulted in little or no induction of accumulation of *RDR1* transcripts after infection by *Potato virus Y* (PVY). Reduced accumulation of *NtRDR1* transcripts also resulted in lower or no induced expression of SA-inducible AOX gene encoding mitochondrial alternative oxidase (Rakshandehroo et al. 2009). These results suggest that RDR1 is involved in both RNA silencing and SA signaling defense pathways and also demonstrate the interplay between these two defense pathways (Fig. 2.15).

A viral suppressor of RNA silencing, 2b of CMV has been found to be an antagonist of the SA-mediated virus resistance mechanism (Ji and Ding 2001).

Systemic infection of CMV-Δ2b (CMV-Δ2b contains a U to A substitution at nucleotide 2,420 of RNA2 that converts the fourth codon of the 2b gene into a stop codon) but not CMV was completely blocked by SA treatment, indicating a direct correlation between Cmv2b expression and the suppression of SA-mediated virus resistance (Ji and Ding 2001). Expression of *cmv2b* was linked tightly to inhibition of SA inducibility of *Aox* (alternative oxidase) (Ji and Ding 2001). These results suggest that a virus-encoded suppressor of RNA silencing protein is able to interfere with the SA-mediated resistance mechanism. An increased in vivo SA accumulation, resulting from CMV infections or an exogenous application, potentiates a systemic RNA silencing antiviral defense response in tobacco plants that is sensitive to 2b VSR protein (Ji and Ding 2001).

2.14 Salicylic Acid Signaling Is Involved in Induction of Systemic Acquired Resistance

Systemic acquired resistance (SAR) is a heightened state of defense against a broad spectrum of pathogens activated throughout a plant following a local infection (Liu et al. 2011a). The induced resistance is detected in the uninoculated systemic tissue of a pathogen-infected plant (Liu et al. 2011b). Localized attack by a necrotizing pathogen induces SAR to subsequent attack by a broad range of normally virulent pathogens. Salicylic acid (SA) accumulation is required for activation of local defenses at the initial site of attack, and in the distant pathogen-free organs for the induction of SAR. SA accumulation and signaling in these distant pathogen-free organs are primed to further increase to higher levels upon challenge with a pathogen (Maldonado et al. 2002; Jung et al. 2009; Návarová et al. 2012; Shah and Zeier 2013).

SA signaling has been shown to be required for the manifestation of SAR (Nandi et al. 2004; Du et al. 2012). Isochorismate synthase activity is required for SA synthesis (Gaille et al. 2002, 2003; Garcion et al. 2008). The *Arabidopsis ics1* mutant, which is deficient in isochorismate synthase 1 activity, has been found to be SAR deficient (Wildermuth et al. 2001; Jung et al. 2009; Chaturvedi et al. 2012). Transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase encoded by the *Pseudomonas putida nahG* gene were found to be deficient in expressing SAR (Vernooij et al. 1994; Lawton et al. 1995). The *FMO1* (*FLAVIN-DEPENDENT MONOOXYGENASE1*) gene is required for the systemic accumulation of SA in distant pathogen-free leaves, and the *FMO1* gene is required for the induction of SAR (Chaturvedi et al. 2012; Shah and Meier 2013). Methyl esterase 1 (StMES1) is required for SAR in potato (Manosalva et al. 2010), and MES is required for accumulation of free SA in the distal systemic tissue, the tissue that does not receive the primary (initial) infection (Park et al. 2007). *NPR1* gene is a master regulator of the SA-mediated induction of defense genes (Mou et al. 2003; Zhang et al. 2003; Chern et al. 2008). NPR1 directly binds SA (Wu et al. 2012) and activates SA signaling system. NPR1 is involved in triggering SAR (Maier et al. 2011; Hermann et al. 2013), and the *npr1* mutant of *Arabidopsis thaliana* is deficient in SAR (Durrant and Dong 2004).

2.15 Mobile Long-Distance Signals for Induction of Systemic Acquired Resistance

2.15.1 Search for Long-Distance Mobile Signal

The establishment of SAR may require translocation of a signal from the pathogen-inoculated leaf to the distal organs, where salicylic acid-dependent defenses are activated (Chaturvedi et al. 2008). Some mobile signal(s) produced at the site of primary infection may have to carry the SAR message and travel throughout the plant. Search for the mobile signals involved in SAR is going on for the past several decades (Yalpani et al. 1991; Vernooij et al. 1994; Park et al. 2007; Truman et al. 2007; Chaturvedi et al. 2008; Jung et al. 2009; Park et al. 2009; Shah 2009; Manosalva et al. 2010; Chanda et al. 2011; Liu et al. 2011a, b; Návarová et al. 2012). It was initially suggested that SA itself may be the mobile signal. Accumulation of SA in the phloem and its requirement to activate SAR led to conclusion that SA may be the mobile signal (Yalpani et al. 1991). However, grafting experiments with tobacco plants expressing the bacterial *NahG* gene, which encodes the SA-degrading enzyme SA hydroxylase, showed that SA may not be the mobile signal. *Tobacco mosaic virus* (TMV)-infected NahG rootstocks were still capable of generating the signal for induction of SAR in wild-type scions, despite their inability to accumulate SA (Vernooij et al. 1994).

Subsequently, several mobile signals have been identified (Fig. 2.16). Methyl salicylate (MeSA) was identified as a mobile signal in inducing SAR (Park et al. 2007, 2009; Vlot et al. 2008a, b; Manosalva et al. 2010). In addition to MeSA, several candidate mobile signals that are linked to lipid metabolism and translocation have been reported (Kachroo et al. 2001; 2004; Nandi et al. 2003, 2004; Chaturvedi et al. 2008; Jung et al. 2009; Chanda et al. 2011). A lipid transfer protein (DIR1), a lipid-derived molecule (glycerol 3-phosphate), and a glycerol-3-phosphate-dependent factor have been reported as lipid-based mobile signaling components in SA-induced SAR. Azelaic acid is the other lipid signal, which is involved in SAR (Jung et al. 2009). An abietane diterpenoid, dehydroabietinal (DA) has been found to be a potent activator of SAR (Chaturvedi et al. 2012). The nonprotein amino acid pipecolic acid, a common Lys catabolite, has also been identified as a systemic signal molecule (Návarová et al. 2012; Shah and Zeier 2013). Thus, several signaling molecules may be involved in long-distance signaling transport to induce SAR (Fig. 2.16).

2.15.2 Methyl Salicylate May Be a Mobile Signal

It has been suggested that methyl salicylate (MeSA) may be a mobile signal involved in SAR. MeSA serves as a long-distance phloem-mobile SAR signal in tobacco and *Arabidopsis* (Manosalva et al. 2010). MeSA is synthesized from salicylate (SA) via a reaction catalyzed by S-adenosyl-L-methionine (SAM): salicylic acid carboxyl

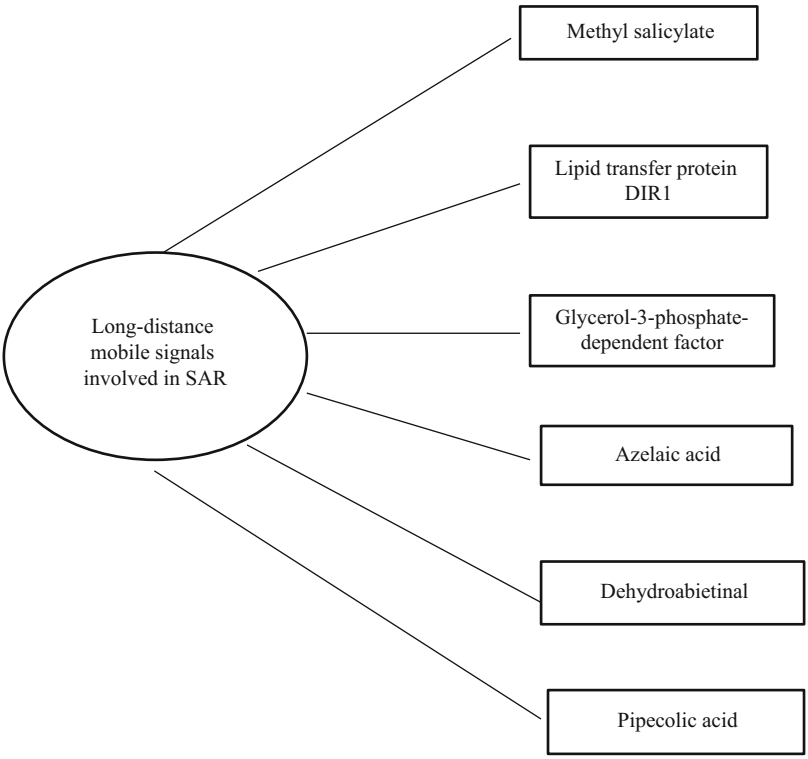

Fig. 2.16 Long-distance mobile signal complex in induction of systemic acquired resistance

methyltransferase (SAMT) with a methyl group donated by the molecule SAM (Kwon et al. 2009). SAMT genes are induced by pathogen or SA treatment (Xu et al. 2006; Koo et al. 2007). Altering expression of SAMT1 compromises SAR (Liu et al. 2010). SAR development appears to require SAMT1 in the primary infected tissue to produce MeSA (Park et al. 2007). The enhanced expression of *SAMT1* gene results in the conversion of total SA to MeSA (Kwon et al. 2009).

MeSA is biologically inactive in induction of defense response. It has been demonstrated that it is a phloem-mobile signal. MeSA moves systemically to the noninfected tissues and is associated with the induced systemic resistance (Kumar and Klessig 2008; Park et al. 2009). By contrast, SA is a highly active signal inducing expression of defense genes, but SA is transported upward only in small amounts via xylem (Rocher et al. 2006). It seems that MeSA which moves systemically through phloem may be converted to active SA form in the distal systemic tissue, the tissue that does not receive the primary (initial) infection (Park et al. 2007, 2009).

A high-affinity SA-binding protein (SABP) termed SABP2 has been identified and characterized from tobacco (Du and Klessig 1997; Kumar and Klessig 2003; Forouhar et al. 2005; Kumar et al. 2006; Tripathi et al. 2010). Orthologs of tobacco

SABP2 have been identified in *Arabidopsis thaliana* (Vlot et al. 2008a, b) and potato (Manosalva et al. 2010). SABP2 is a lipase belonging to the α/β hydrolase superfamily. It is present in extremely low abundance and specifically binds SA with high affinity (Kumar and Klessig 2003). It has strong esterase activity with methyl salicylate as the substrate (Forouhar et al. 2005). Thus, SABP2 is a methyl salicylate esterase whose function is to convert biologically inactive methyl salicylate to active SA (Kumar and Klessig 2008; Vlot et al. 2008a, b; Manosalva et al. 2010). SABP2's esterase activity is inhibited in the initially primary infected tissue by SA binding in its active site; this facilitates accumulation of methyl salicylate, which is then translocated through the phloem to systemic tissue for perception and processing by SABP2 to SA (Kumar and Klessig 2008; Park et al. 2009). It has been demonstrated that the tobacco SAMT (NtSAMT1) activity, and thus MeSA biosynthesis, is required in the primary infected leaves where the SAR signal is produced. In contrast, MeSA esterase (MSE) activity is needed in the uninoculated systemic leaves, where the SAR signal is perceived and processed (Park et al. 2007). MeSA does not induce defense responses (Seskar et al. 1998), and it must be converted to SA by an MSE for biological activity. The SABP2's MSE activity must be inhibited in the primary infected tissue (by SA binding in its active site pocket) to facilitate the accumulation of sufficient levels of MeSA to signal SAR (Park et al. 2007, 2009). A synthetic SA analog (2,2,2,2′-tetra-fluoroacetophenone) that inhibits MSE activity blocks SAR development in tobacco, potato, and *Arabidopsis* (Park et al. 2009). Collectively these studies suggest that MeSA is a critical mobile signal and on conversion of MeSA by MSE to SA, SA, and not MeSA, activates the expression of defense genes (Fig. 2.17).

Attaran et al. (2009) showed that MeSA production is not essential for induction of systemic acquired resistance (SAR) in *Arabidopsis*. MeSA production increased in *Arabidopsis* leaves inoculated with *Pseudomonas syringae*; however, most MeSA was emitted into the atmosphere, and only small amounts were retained. In several *Arabidopsis* defense mutants, the abilities to produce MeSA and to establish SAR did not coincide. T-DNA insertion lines defective in expression of a pathogen-responsive SA methyltransferase gene were completely devoid of induced MeSA production but increased systemic SA levels and developed SAR upon local *P. syringae* inoculation (Attaran et al. 2009). Further, it has been demonstrated that MeSA production induced by *P. syringae* depended on the JA pathway but the JA biosynthesis or downstream signaling was not required for SAR (Attaran et al. 2009). These results suggested that MeSA is dispensable for SAR in *Arabidopsis*.

However, Liu et al. (2011b) showed that the length of light exposure that plants receive after the primary infection determined the extent to which MeSA is required for SAR signaling. When the primary infection occurred late in the day and as a result infected plants received very little light exposure before entering the night/dark period, MeSA and its metabolizing enzymes were essential for SAR development. In contrast, when infection was in the morning followed by 3.5 h or more of exposure to light, SAR developed in the absence of MeSA. These studies suggest that length of light exposure that plants receive after the primary infection may determine the role of MeSA in SAR.

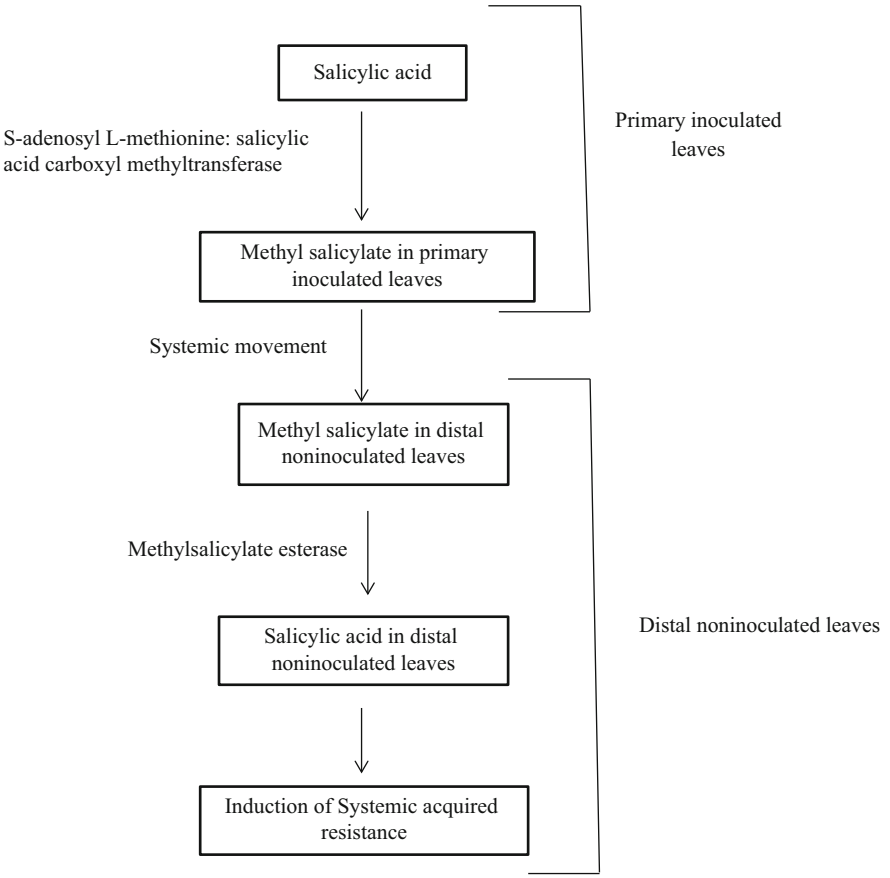

Fig. 2.17 Role of salicylic acid carboxyl methyltransferase, methyl salicylate, and methyl salicylate esterase in induction of systemic acquired resistance (Adapted from Park et al. 2007, 2009; Kwon et al. 2009)

2.15.3 DIR1 and Glycerol-3-Phosphate-Dependent Factor Mobile Signal Complex

Maldonado et al. (2002) reported another mobile signal molecule involved in SAR in *Arabidopsis*. They characterized an *Arabidopsis* mutant, *dir1-1* (*D*efective in *I*nduced *R*esistance 1-1), which is specifically compromised in SAR. These plants contained a mutation in a putative apoplastic protein with homology to family 2 lipid transfer proteins, and they are compromised for SAR but not for local resistance. The *dir1-1* mutant exhibited wild-type local resistance to avirulent and virulent *Pseudomonas syringae*, but pathogenesis-related (PR) gene expression was abolished in uninoculated distant leaves, and *dir1-1* failed to develop SAR to virulent *Pseudomonas syringae* or *Peronospora parasitica* (Maldonado et al. 2002).

The results suggest that the *dir1-1* is defective in the production or transmission from the inoculated leaf of an essential mobile signal.

Glycerol 3-phosphate (G3P) has been shown to be an inducer of SAR. Genetic mutants defective in G3P biosynthesis cannot induce SAR but can be rescued when G3P is supplied exogenously (Chanda et al. 2011). Radioactive tracer experiments showed that a glycerol-3-phosphate derivative was translocated to distal tissues and this translocation required the lipid transfer protein, DIR1. It was also observed that glycerol 3-phosphate was required for the translocation of DIR1 to distal tissues, which occurs through the symplast. The *dir1* mutant plants accumulated reduced levels of glycerol 3-phosphate (Chanda et al. 2011). These results suggest the cooperative interaction of the lipid transfer protein (DIR1) and the lipid-derived molecule (glycerol 3-phosphate) orchestrates the induction of SAR in plants (Maldonado et al. 2002).

A plastid glycerol-3-phosphate-derived factor has also been shown as a mobile signal molecule. Petiole exudates (PeXs) collected from *Arabidopsis* leaves inoculated with an avirulent (Avr) *Pseudomonas syringae* strain promoted resistance when applied to *Arabidopsis*, tomato, and wheat (Chaturvedi et al. 2008). *Arabidopsis* FATTY ACID DESATURASE7 (FAD7), SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 (SFD1), and SFD2 genes have been shown to be required for accumulation of the SAR-inducing activity. In contrast to Avr PeX from wild-type plants, Avr PeXs from *fad7*, *sfd1*, and *sfd2* mutants were unable to activate SAR when applied to wild-type plants (Chaturvedi et al. 2008). The *sfd1* mutation diminished the SAR-associated accumulation of elevated levels of SA and *PR1* gene transcript in the distal leaves of plants previously inoculated with an avirulent pathogen (Nandi et al. 2004). FAD7, SFD1, and SFD2 are known to be involved in plastid glycerolipid biosynthesis (Chaturvedi et al. 2008). The *sfd1* mutants had defects in lipid composition; in particular levels of the plastid-localized 34:6-monogalactosyldiacylglycerol were lower in the *sfd1* mutant (Chaturvedi et al. 2008, 2012).

Biologically induced SAR was compromised in the *sfd1* mutant (Chaturvedi et al. 2008). *SFD1* encodes a plastid-localized dihydroxyacetone phosphate (DHAP) reductase that synthesizes glycerol 3-phosphate (G3P). SFD1's DHAP reductase activity and its localization to the plastids, suggesting that SFD1 synthesized G3P, or a product thereof, are required for the accumulation and long-distance transport of a SAR signal (Lorenc-Kukula et al. 2012; Shah and Zeier 2013). In *Arabidopsis*, G3P levels were elevated in the pathogen-inoculated and the distal pathogen-free leaves (Chanda et al. 2011). Locally applied ^{14}C-labeled G3P could not be recovered in the systemic leaves (Chanda et al. 2011). The result suggests that G3P per se is unlikely to be the systemically translocated SAR signal and rather, a G3P-dependent factor is likely involved in long-distance signaling (Shah and Zeier 2013).

SFD1- and DIR1-dependent factors may function together in long-distance signaling. G3P, when co-applied with DIR1 protein, was capable of enhancing systemic disease resistance (Chanda et al. 2011). G3P levels were lower in *dir1* mutant, suggesting that the DIR1 and the G3P-dependent factor are required for systemic translocation of each other (Chanda et al. 2011).

2.15.4 Azelaic Acid May Be a Mobile Signal

A nine-carbon dicarboxylic acid, azelaic acid, has been found to accumulate in the vascular sap of *Arabidopsis* plants infected by the bacterial pathogen *Pseudomonas syringae* (Jung et al. 2009). Levels of azelaic acid increased in plants exposed to pathogens and triggered systemic acquired resistance (Zahn 2009). Azelaic acid had several properties of a long-distance resistance-priming signal (Park et al. 2009). The accumulated azelaic acid primed plants to accumulate SA upon infection by the pathogen. Azelaic acid induced the expression of *AZI1* (for *Azelaic acid induced 1*) gene. Mutation of the *AZI1* gene resulted in the specific loss of SAR triggered by azelaic acid (Jung et al. 2009). The results suggest that the predicted secreted protein AZI1 is important for generating vascular sap that confers disease resistance. The azelaic acid induced local and systemic resistance against *P. syringae* in *Arabidopsis* (Jung et al. 2009).

Azelaic acid may be synthesized by oxidation of 9-oxononanoic acid synthesized from fatty acids by the action of 9-lipoxygenase and hydroperoxide lyase (Vicente et al. 2012). The importance of azelaic acid as the systemic molecule in inducing SAR is doubtful. It has been shown that azelaic acid is not essential for the establishment of SAR per se (Návarová et al. 2012; Zoeller et al. 2012). However, when azelaic acid is translocated, it can add to the strength of systemic immunity observed during SAR (Shah and Zeier 2013).

2.15.5 Dehydroabietinal as a Mobile Signal

Dehydroabietinal (DA), an abietane diterpenoid, has been found to be an activator of SAR (Chaturvedi et al. 2012). It is a potent inducer of SAR and it is active when applied as picomolar solutions to leaves of *Arabidopsis*, tomato, and tobacco. DA was purified from vascular sap of *Arabidopsis thaliana* leaves treated with SAR-inducing microbe. Deuterated DA when applied to *Arabidopsis* leaves was rapidly transported out of the leaf and recovered from the untreated leaves (Chaturvedi et al. 2012). Locally applied DA was translocated throughout the plant and systemically induced the accumulation of SA and *PR1* expression in the untreated leaves. DA-induced SAR was attenuated in the SA-deficient *NahG* transgenic, *ics1 ics2* double mutant, and *npr1* mutant plants. It suggests that DA functions upstream of SA accumulation and signaling. *FMO1* gene has been shown to be required for systemic SA accumulation in DA-treated plants and DA-induced SAR (Chaturvedi et al. 2012; Shah and Zeier 2013). DA in vascular sap was redistributed into a SAR-inducing "signaling DA" pool that was associated with a trypsin-sensitive high-molecular-weight fraction (Chaturvedi et al. 2012). It suggests that DA-orchestrated SAR involves a vacular sap protein(s). However, the proteins involved in this process have not yet been characterized.

2.15.6 *Pipecolic Acid as an SAR Long-Distance Signal*

SA accumulates locally at site of the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) infection and also in distal noninoculated leaves (Návarová et al. 2012). SA was not transported from inoculated to distal leaves, but it systemically accumulated in the distal leaves (Vernooij et al. 1994). Systemic SA accumulation proceeds via regulation of *ICS1* and de novo SA biosynthesis (Attaran et al. 2009). *ALD1* (*AGD2-LIKE DEFENSE RESPONSE PROTEIN1*) and *FMO1* (*FLAVIN-DEPENDENT MONOOXYGENASE1*) are also involved in systemic SA accumulation and SAR (Mishina and Zeier 2006; Návarová et al. 2012). Pathogen-induced expression of *ALD1*, *FMO1*, and *ICS1* in inoculated tissue proceeds independently of SA (Metraux 2002; Song et al. 2004a, b; Bartsch et al. 2006; Mishina and Zeier 2006).

The nonprotein amino acid pipecolic acid (Pip) has been identified as a mobile long-distance signal in the SAR induction pathway. Pip strongly accumulated in *Arabidopsis* leaves inoculated with the SAR-inducing *Pseudomonas syringae* pv. *maculicola* (*Psm*) and in leaves treated with the bacterial PAMPs flg22 and LPS (Návarová et al. 2012). It also accumulated in distal, noninoculated leaves of *Psm-treated* plants, and there was tenfold systemic increase at 48 h after local *Psm* inoculation (Návarová et al. 2012).

The SA accumulation in distal leaves may be triggered by perception of mobile long-distance signals (Shah 2009). Significantly enhanced systemic levels of Pip but not of SA were observed at 36h after *Psm* inoculation, suggesting that Pip accumulation precedes SA accumulation in systemic leaves at the onset of SAR. Systemic Pip levels start to significantly rise before marked elevations of SA are detectable in the systemic tissue (Návarová et al. 2012). Systemic accumulation of Pip was markedly reduced in the SAR-defective *fmo1* and *ics1* mutants; localized bacterial treatment still provoked significant increases of Pip in distal leaves of these mutants. By contrast, systemic levels of Pip were not enhanced in the *npr1* and *pad4* mutants (Návarová et al. 2012).

ALD1, an aminotransferase with Lys converting activity, may be involved in Pip biosynthesis. ALD1 is important for the activation of both local and systemic defenses in *Arabidopsis* (Song et al. 2004a). ALD1 has aminotransferase activity with strong substrate preference for Lys (Song et al. 2004b). Δ1-Piperideine-2-carboxylic acid and ε-amino-α-ketocaproic acid are direct reaction products of an ALD1-catalyzed Lys aminotransferase reaction. However, it is not yet known how Lys transamination products are converted to Pip (Návarová et al. 2012). *ALD1* transcript levels strongly increased in *Psm*-inoculated plants in both local and distal leaves (Návarová et al. 2012).

Pipecolate-deficient *ald1* plants fail to accumulate SA in distal leaf tissue following pathogen inoculation and are fully compromised in SAR (Jing et al. 2011; Návarová et al. 2012). The *ald1* plants regain the ability for systemic SA accumulation and SAR establishment, when Pip is exogenously applied to the whole plant prior to pathogen treatment. The results suggest that Pip accumulation is critical for

systemic SA production and SAR (Návarová et al. 2012). Pip feeding of plants prior to inoculation boosts pathogen-triggered induction of SA biosynthesis and defense-related gene expression, suggesting that Pip strongly amplifies pathogen-triggered defense responses. It has been suggested that the early systemic increase of Pip at the onset of SAR functions as an initial trigger for signal amplification leading to the systemic increase in SA (Návarová et al. 2012).

FMO1 (Flavin-dependent monooxygenase1) has been identified as an important regulator of SAR. Localized *P. syringae* inoculation triggered enhanced expression of several hundred genes in the distal leaves of *Arabidopsis* plants. This massive switch in gene expression at the systemic plant level is totally lost in the *fmo1* mutant (Mishina and Zeier 2006). *FMO1* is necessary for the systemic accumulation of SA upon SAR induction (Mishina and Zeier 2006). The *fmo1* mutant fails to establish Pip-induced resistance to bacterial infection. These results suggest that FMO1 functions downstream of Pip and upstream of SA in SAR (Návarová et al. 2012). FMO1 may be involved in the oxidation of Pip in the Pip signal amplification pathway (Návarová et al. 2012). *Fmo1* is necessary for systemic resistance induction (Liu et al. 2011a). Pip enhances both its own biosynthesis and downstream signaling in SAR via amplification of pathogen-triggered *ALD1* and *FMO1* expression, indicating a positive feedback amplification loop with Pip as a central player (Návarová et al. 2012; Shah and Zeier 2013).

PAD4 (Phytoalexin-deficient4) and NPR1 are also involved in Pip-mediated resistance (Mishina and Zeier 2006; Jing et al. 2011). PAD4 promotes pathogen-induced Pip production, and it is also required for resistance promoted by Pip application (Návarová et al. 2012). PAD4 seems to exert its central defense regulatory role via transcriptional control of *ALD1*, *FMO1*, and *ICS1* (Shah and Zeier 2013). A substantial increase of Pip biosynthesis was observed 10 h after the challenge infection in SAR-induced plants, indicating a strong potentiation of Pip biosynthesis during SAR (Návarová et al. 2012).

SA content increases in the distal leaves of plants that were inoculated with pathogen on other leaves. SA content increase was fully dependent on *ALD1-mediated* Pip biosynthesis and downstream signaling gene *FMO1* (Song et al. 2004a; Mishina and Zeier 2006; Návarová et al. 2012; Shah and Zeier 2013). The results suggest that Pip triggers de novo biosynthesis of SA on distal leaves. Conversely, systemic Pip accumulation in the distal leaves strongly relies on *FMO1* and *ICS1*-mediated SA biosynthesis (Návarová et al. 2012). The results suggest that strong SAR establishment depends on effective signal amplification involving feedback mechanisms that integrate Pip and SA signaling (Fig. 2.18; Shah and Zeier 2013).

Pip also induces priming of SAR responses. Pip application to *Arabidopsis* plants significantly increased *ALD1* transcript levels and strongly primed plants for pathogen-triggered expression of *ALD1*, indicating a positive regulatory role for Pip on its own biosynthesis. Pip feeding also led to strong priming of *Psm*-triggered *FMO1* and *PR1* gene expression (Návarová et al. 2012). Pip-deficient *ald1* mutant is defective in SAR priming, and it suggests that endogenously accumulating Pip mediates priming during SAR (Návarová et al. 2012).

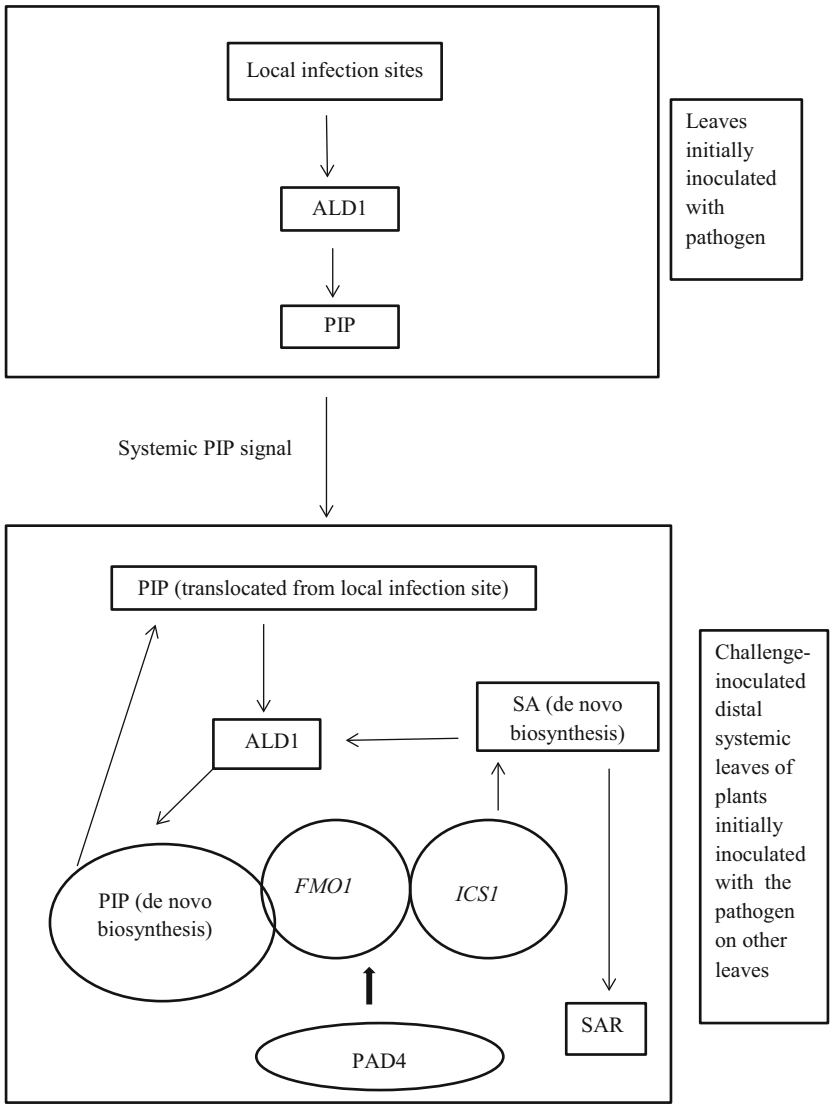

Fig. 2.18 Feedback amplification of Pip and SA in the SAR pathway (Adapted from Návarová et al. 2012; Shah and Zeier 2013)

2.16 Role of Mediator Complex in SA-Mediated Systemic Acquired Resistance

Some Mediators have been shown to be involved in triggering SA-mediated SAR. Mediator is a multiprotein complex that functions as a transcriptional coactivator (Kidd et al. 2011; An and Mou 2013). Mediator fine-tunes gene-specific and

pathway-specific transcriptional reprogramming by interacting with specific activators/repressors together with general transcription factors at the promoter site (Conaway and Conaway 2011a, b; An and Mou 2013). Mediator performs both general and specific roles in regulating gene transcription. Mediator subunits may regulate a wide range of signaling pathways through selectively interacting with specific transcription factors (Cevik et al. 2012; Chen et al. 2012; Wathugala et al. 2012).

The *Arabidopsis* Mediator subunit16 (MED16) has been shown to be an essential positive regulator of SAR (Zhang et al. 2012b). Mutations in MED16 reduced NPR1 protein levels (Zhang et al. 2012b). The transcription co-activator NPR1 is the master regulator of SAR in *Arabidopsis* (Maier et al. 2011; Shah and Zeier 2013), and MED16 may regulate the function of NPR1 in inducing SAR. MED16 might regulate SA responsiveness via the modulation of NPR1 protein accumulation. Following *Pseudomonas syringae* inoculation, *med16* mutant plants locally and systemically accumulated SA to levels similar to that in wild-type plants but impaired in PR gene expression (Zhang et al. 2012b). The results suggest that MED16 may act downstream of SA signaling and upstream of NPR1 in SAR signaling pathway (Fig. 2.19; Zhang et al. 2012b). It is suggested that MED16 might relay signals from the SA pathway to the transcription machinery. *Med16* knockout lines exhibited increased susceptibility to both avirulent and virulent strains of *P. syringae* and were unable to establish SAR (Zhang et al. 2012b). The Mediator subunit SFR6/MED16 in *Arabidopsis* has been shown to be involved in conferring resistance against *P. syringae*. The *srf6* mutants were more susceptible to *P. syringae*. They exhibited weaker

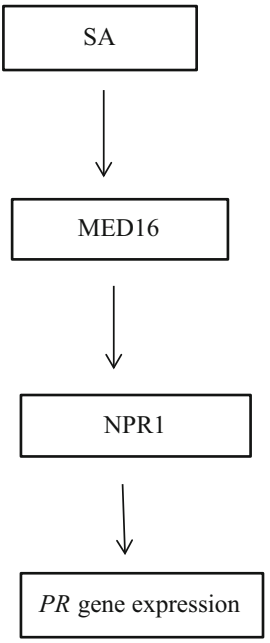

Fig. 2.19 MED16 acts downstream of SA and triggers NPR1 accumulation to trigger *PR* gene expression (Adapted from Zhang et al. 2012b)

expression of the SAR marker *PR* gene than wild-type *Arabidopsis* after direct application of SA (Wathugala et al. 2012). These results suggest that MED16 plays an important role in induction of SAR.

The Mediator NRB4 (Non-recognition-of-BTH4) has been shown to be involved in SAR in *Arabidopsis* (Canet et al. 2012). NRB4 is allelic to Mediator subunit 15 (MED15). Plants carrying weak *nrb4* alleles exhibit strong insensitivity and show attenuated SAR to *P. syringae*. Like *npr1* mutant, *nrb4* mutants fail to develop SA-induced resistance and overaccumulate SA during the *P. syringae* infection process (Canet et al. 2012). NRB4 may function downstream of NPR1 in SA signaling pathway. Overexpression of NPR1 in *med15/nrb4* mutant does not restore the response to SA. It has been shown that MED15/NRB4 is not required for NPR1 stability and subcellular localization and no protein–protein interaction exists between MED15 and NPR1 (Canet et al. 2012). The results suggest that MED15/NRB4 may act downstream of NPR1 in regulating SA response.

2.17 Salicylic Acid Triggers Priming and Induces Systemic Acquired Resistance

2.17.1 What Is SA-Triggered Priming?

Infection of plants by necrotizing pathogens, which induce the accumulation of SA, or treatment of plants with synthetic compounds, which are able to trigger SA signaling, causes the induction of a unique physiological state called "priming" (Conrath 2009, 2011; Camañes et al. 2012; Slaughter et al. 2012). When a treatment puts a plant in a state of increased alertness with no or only minimal gene induction, it is called priming (Conrath et al. 2002, 2006). After localized foliar infections by diverse pathogens or by treatment with synthetic compounds, plants develop whole-plant immunity, called SAR (Conrath et al. 2006; Beckers and Conrath 2007; Conrath 2009; Mukherjee et al. 2010). In this process, distal (systemic) leaves become primed to activate stronger defense response upon secondary infection (Beckers et al. 2009; Slaughter et al. 2012). SAR is associated with priming of defense (Kohler et al. 2002; Jung et al. 2009; Luna et al. 2012), and the priming results in a faster and stronger induction of defense mechanisms after pathogen attack (Conrath 2011). The potentiated induction of defense responses only becomes apparent after further pathogen challenge and has often been overseen when studying SAR (Kohler et al. 2002; Conrath et al. 2006; Ahmad et al. 2010). Priming of pathogen-induced gene allows the plant to react more effectively to a subsequent invader (Conrath et al. 2002, 2006; Conrath 2009). In contrast to constitutive activation of defense responses, priming does not require major metabolic changes when no pathogens are present. Therefore, it forms a low-cost defense strategy while acting against a broad

spectrum of attackers (Van Hulten et al. 2006; Pieterse and Dicke 2007; Van der Ent et al. 2008).

This defense priming offers disease protection that is effective against a broad spectrum of virulent pathogens (Ahmad et al. 2010). SA or its functional analog benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), an SA biosynthesis-inducing chemical probenazole, and a nonprotein amino acid β-aminobutyric acid are known to induce priming and confer resistance against viral (Mandal et al. 2008), bacterial (Flors et al. 2008), oomycete (Hamiduzzaman et al. 2005; Ziadi et al. 2008), and fungal (Hukkanen et al. 2007; Umemura et al. 2009) diseases.

2.17.2 Accumulation of Dormant MAPKs May Be Involved in SA-Triggered Priming

Although the priming phenomenon has been known for decades, the molecular basis of SA-triggered priming is poorly understood (Conrath 2011). Mitogen-activated protein kinase (MAPK) cascades are major pathways downstream of sensors/receptors that transduce extracellular stimuli into intracellular responses in plants (Liu et al. 2003; Pedley and Martin 2005; Hettenhausen et al. 2012; Zhang et al. 2012a). The MAPKs transduce extracellular stimuli into intracellular transcription factors through activation of Ca^{2+}, ROS, SA, JA, and ethylene-dependent signaling systems and enhance expression of defense-related genes in plant innate immune system (Vidhyasekaran 2014). Some dormant MAPKs have been suggested to be important components required for priming in *Arabidopsis*, and the prestress deposition of these inactive kinases may be a possible mechanism of priming during the development of systemic acquired resistance (Beckers et al. 2009). MPK3 and functionally redundant MPK6 have been found to be important components for full priming in *Arabidopsis*. The resistance-inducing avirulent strains of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* induced SA accumulation and MPK3 expression. Both SA and the SA-related compounds benzothiadiazole (BTH) and 4-chloro-SA activate *MPK3* gene expression and induce priming and SAR. In contrast, another SA-related compound 3-hydroxybenzoic acid did not induce *MPK3* gene expression, priming, and disease resistance. This strong correlation between the ability of avirulent bacteria and various SA-related compounds to activate *MPK3* gene expression and their capacity to prime plants for augmented defense gene activation and induced resistance suggests that MPK3 plays a role in priming (Beckers et al. 2009). Similarly another MAPK gene, *MPK6*, was also found to be involved in priming process. However, the BTH-induced accumulation of *MPK6* transcript and protein was less pronounced. Both MPK3 and MPK6 accumulate in an inactive form during priming of *Arabidopsis* with BTH (Beckers et al. 2009). Both MPK3 and MPK6 displayed greater activity in *Arabidopsis* plants which are primed and subsequently challenged with the virulent *P. syringae* pv. *maculicola*. These two enzymes

were more strongly activated in primed plants than in nonprimed plants. Priming of defense gene expression and induced resistance were lost or reduced in *mpk3* or *mpk6* mutants (Beckers et al. 2009). These results suggest that prestress deposition of the signaling components MPK3 and MPK6 is a critical step in priming plants for full induction of defense responses during induced resistance.

2.17.3 Histone Modifications May Be Involved in Gene Priming in SA-Induced SAR

Chromatin is the combination of DNA and proteins that make up the contents of the nucleus of a cell. Chromatin structure is important for the regulation of gene expression (Ouyang and Gill 2009; Jaskiewicz et al. 2011). The primary function of chromatin is to package DNA into a smaller volume to fit in the cell. The first level of compaction is achieved by nucleosomal packaging of DNA. Each nucleosome comprises 147 bp of DNA wrapped around a histone octamer that consists of two molecules each of histone proteins H2A, H2B, H3, and H4 (Probst et al. 2003, 2004; Shi and Fang 2011). The primary protein components of chromatin are histones that compact the DNA (Bender 2004).

The histone proteins are subject to various covalent modifications including acetylation, methylation, phosphorylation, and ADP-ribosylation (Berger 2002; Britton et al. 2011; Chen et al. 2011). Acetylation of lysines in the amino-terminal tails of histones H3 and H4 has been associated with active genes (Eberharter and Becker 2002). Lysine acetylation is an important modification involved in the regulation of gene expression (Arif et al. 2010). In histone methylation, lysine and arginine residues are methylated, and up to three methyl groups are added to each residue. Specific methylation patterns are associated with gene activation and repression. The strongest correlation between histone methylation and gene activity has been reported for trimethylation of Lys4 on histone H3 (H3K4me3) on promoters and coding sequences of active genes (Ruthenburg et al. 2007).

Jaskiewicz et al. (2011) showed that histone modifications are systemically set during a priming event. These modifications might create a memory of the primary infection that is associated with an amplified reaction to a second stress condition. It is known that chromatin states could control cellular memory (Zhang 2008). The primed genes may be poised for enhanced activation of gene expression by histone modification (Jaskiewicz et al. 2011).

In the SAR response, defense genes in the infected and distant tissue show the "priming" phenomenon; they are able to respond faster and/or to a greater extent to a subsequent challenge (Kohler et al. 2002; Conrath 2009). The promoters of many of these genes contain at least one "W-box" that provides binding sites for WRKY transcription factors (Rushton et al. 2010). Genes encoding WRKY factors are themselves transcriptionally induced by either pathogen infection or treatment with PAMPs (Dong et al. 2003).

The SA analog BTH induces priming (Kohler et al. 2002; Beckers et al. 2009). Priming of defense genes for amplified response to secondary stress can be induced by application of salicylic acid or its synthetic analogue acibenzolar-*S*-methyl. BTH treatment induced a typical priming effect on *WRKY29*, *WRKY6*, and *WRKY53* genes (Jaskiewicz et al. 2011). The expression of the *WRKY* genes was enhanced in BTH-treated plants when the plants were stressed. Water infiltration, a stress-inducing cell death after BTH treatment, resulted in strongly enhanced gene activation, compared with plants that were stressed without previous BTH treatment. On the WRKY29 promoter, trimethylation (H3K4me3) and dimethylation (H3K4me2) of H3K4 and all acetylations tested increased after BTH application although this did not induce *WRKY29* transcription. Thus, chromatin marks normally associated with active genes are set by the priming stimulus before gene activation. After previous priming, a stress stimulus enhanced some of the modifications – H3K4me2, H3K9ac, and H4K12ac – on WRKY29 (Jaskiewicz et al. 2011). Collectively, these results suggest that prestress application of BTH induced chromatin modifications on *WRKY* gene promoters that facilitate the activation of gene expression by subsequent stress (Jaskiewicz et al. 2011).

Localized *Pseudomonas syringae* pv. *maculicola* infection primed the *WRKY* gene promoters in remote leaves to an augmented response to secondary stress, and the transcriptional responses in distal leaves were similar to those observed with BTH. On the three *WRKY29*, *WRKY6*, and *WRKY53* gene promoters, clear increases in histone modifications were observed after pathogen infection. The response amplitude after perception of the systemic signals for SAR was similar to that observed after BTH treatment. Thus, pathogen exposure induces one or more systemic signals that are stored in the form of histone modifications. Enhanced trimethylation of H3K4 concomitant with gene priming was a common feature of the assayed *WRKY* promoters (Jaskiewicz et al. 2011). Collectively, these studies suggest that there may be a tight correlation between histone modification patterns and gene priming and also there may be a histone memory for information storage in the plant stress response (Jaskiewicz et al. 2011).

2.17.4 NPR1 May Be Involved in Chromatin Modification-Induced Priming

Mutants that are attenuated in pathogen defense are often also compromised in gene priming. The *npr1* mutant of *Arabidopsis thaliana* is deficient in SAR (Durrant and Dong 2004) and cannot be primed for enhanced gene expression (Kohler et al. 2002; Beckers et al. 2009). BTH induced trimethylation of H3K4 on the *WRKY29* promoter in the wild-type *Arabidopsis thaliana* plants, but not on the priming-deficient *npr1* mutant. The results were similar to *WRKY6* and *WRKY53* expression and histone modification. Neither gene augmented expression after BTH treatment and stress stimulus in the *npr1* mutant. This correlated with the impaired ability of *npr1* to induce high H3K4me3 levels on the *WRKY6* and *WRKY53* promoters in response to BTH (Jaskiewicz et al. 2011). The results suggest that NPR1 plays important role in inducing high levels of chromatin modification on promoters of WRKY genes.

2.17.5 Histone Replacement May Be Instrumental for Priming of SA-Responsive Loci

Salicylic acid is known to trigger a long-lasting systemic acquired resistance (SAR). SAR involves extensive reprogramming of transcription (Thibaud-Nissen et al. 2006; Wang et al. 2006). SA mediates changes in the expression pattern of about 1,000–2,000 genes. Such a broad effect on gene transcription may be associated with extensive chromatin remodeling, which would require the involvement of specific chromatin remodeling complexes (March-Diaz et al. 2008). The chromatin remodeling may involve substitution of canonical histones in the octamer by histone variants, in a process known as histone replacement (Kamakka and Biggins 2005). One of the histone variants, H2A.Z, has been linked to both transcriptional repression and activation. Histone H2A.Z is incorporated into chromatin through the action of a multisubunit complex termed SWR1 in yeast. *Arabidopsis* PIE1 (for PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1) is homologous to the yeast SWR1 protein. The histone variant H2A.Z, but not canonical H2A histone, interacts with PIE1. N-terminal region of *PIE1* specifically interacts with *Arabidopsis* H2A.Z histone variants. A role for PIE1 and histone H2A.Z has been observed in the priming process in SAR (March-Diaz et al. 2008).

To study the role of PIE1 in SAR, *pie1* mutant plants were developed. A total of 622 genes were differentially expressed between *pie1* mutant and wild-type (WT) plants. Most of the mis-regulated genes in *pie1* plants were related to SAR response. Several genes mis-regulated in *pie1* plants were also mis-regulated in wild-type plants treated with an analogue of SA, benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH), which induces SAR (March-Diaz et al. 2008). These results suggest that PIE1 plays an important role in SA-mediated SAR.

The *pie1* mutant plants showed increased expression of several genes involved in SA accumulation. The level of *EDS5* mRNA increased in *pie1* mutant plants. EDS5 is involved in triggering SA accumulation. The amount of ICS1 mRNA increased about twofold in the absence of PIE1. ICS1 (also called SID2) encodes isochorismate synthase 1, an enzyme that is required for the synthesis of SA. PIE1 may also regulate downstream of the SA signal regulating NPR1 targets (March-Diaz et al. 2008). *pie1* mutant plants also showed increased resistance to virulent *Pseudomonas syringae* pv. *tomato*.

Comparison of gene expression profiles revealed that 65 % of the genes were misregulated in *pie1* mutant plants and the majority of mis-regulated genes were related to SA-dependent immunity. Several SAR marker genes were constitutively expressed in *pie1* plants. Such variations observed at the molecular level resulted in enhanced resistance to *Pseudomonas syringae* pv. *tomato*. Collectively these results suggest that PIE1 chromatin remodeling complex maintains a repressive state of the SAR response and this response is related to priming (March-Diaz et al. 2008). When PIE1 is inactivated, SAR is induced. It may be possible that defense genes are poised for enhanced activation during SAR by replacement on gene promoters of histone H2A with its variant H2A.Z (March-Diaz et al. 2008). Chromatin remodeling

may be instrumental for priming of SA-responsive loci to enable their enhanced reactivation upon subsequent pathogen attack (van den Burg and Takken 2009).

2.18 Next-Generation Systemic Acquired Resistance

The priming can be inherited epigenetically from disease-exposed plants, and descendants of primed plants exhibit next-generation systemic acquired resistance (Luna et al. 2012; Slaughter et al. 2012). Slaughter et al. (2012) compared the reactions of *Arabidopsis thaliana* plants that had been either primed with an avirulent isolate of *Pseudomonas syringae* pv. *tomato* or primed with β-aminobutyric acid (BABA). The descendants of primed plants showed a faster and higher accumulation of transcripts of defense-related genes in salicylic acid signaling pathway and enhanced disease resistance upon challenge inoculation with a virulent isolate of *P. syringae*. The progeny of the primed plants was also more resistant against the oomycete pathogen *Hyaloperonospora arabidopsidis*. When transgenerationally primed plants were subjected to an additional priming treatment, their descendants displayed an even stronger primed phenotype (Slaughter et al. 2012). The results suggest that plants can inherit a sensitization for the priming phenomenon and the primed state of plants is transferred to their progeny.

The transgenerational SAR was found to be sustained over one stress-free generation, indicating an epigenetic basis of the phenomenon (Luna et al. 2012). The progeny from *Pseudomonas syringae* pv. *tomato*-inoculated *Arabidopsis thaliana* plants (P_1) displayed reduced responsiveness of jasmonic acid (JA)-inducible genes and enhanced susceptibility to the necrotrophic fungus *Alternaria brassicicola* (Luna et al. 2012). JA signaling is known to activate defense mechanisms against necrotrophic pathogens (McGrath et al. 2005; Zheng et al. 2006; Jakob et al. 2007; Korolev et al. 2008; Pré et al. 2008), and such signaling is suppressed in the P1 progeny which shows enhanced expression of SA-inducible genes. This shift in SA and JA responsiveness was not associated with changes in corresponding hormone levels. Instead, SA-inducible promoters of *PR1*, *WRKY6*, and *WRKY53* in P1 progeny plants were enriched with acetylated histone H3 at lysine 9, a chromatin mark associated with a permissive state of transcription. In contrast, the JA-inducible promoter of PDF1.2 showed increased H3 triple methylation at lysine 21, a mark related to repressed gene transcription (Luna et al. 2012). The results suggest the importance of chromatin remodeling in the transgenerational SAR. P1 progeny from defense regulatory mutant *npr1-1* failed to develop transgenerational defense phenotypes (Luna et al. 2012). It demonstrates the important role for NPR1 in expression of transgenerational defense responses.

DNA methylation may also play important role in transgenerational SAR (Luna et al. 2012). DNA methylation is initiated by DRM (Domains-Rearranged methyltransferase) and CMT3 (Chromomethyltransferase3) that are methyltransferases (Cao et al. 2003; Henderson et al. 2010; Huang et al. 2010; Greenberg et al. 2011; Naumann et al. 2011). Neither *drm* nor *cmt3* mutants affected the maintenance of preestablished RNA-directed CpG methylation. However, *drm* mutants showed a

nearly complete loss of asymmetric methylation and a partial loss of CpNpG methylation. The remaining asymmetric and CpNpG methylation was dependent on the activity of CMT3, showing that DRM and CMT3 act redundantly to maintain non-CpG methylation. These DNA methyltransferases appear to act downstream of siRNAs, since *drm1 drm2 cmt3* triple mutants show a lack of non-CpG methylation but elevated levels of siRNAs. Further it has been demonstrated that DRM activity is required for the initial establishment of DNA methylation in all sequence contexts including CpG, CpNpG, and asymmetric sites (Cao et al. 2003).

The *drm1 drm2 cmt3* triple mutant has been shown to be affected in non-CpG DNA methylation. This mutant mimicked the transgenerational SAR. The bacterial pathogen *P. syringae* pv. *tomato* induces DNA hypomethylation in *Arabidopsis* (Luna et al. 2012). Collectively these results suggest that transgenerational SAR is transmitted by hypomethylated genes that direct priming of SA-dependent defenses in the following generations.

2.19 Crosstalk Between Salicylate and Jasmonate Signaling Systems

2.19.1 Antagonism Between SA and JA Signaling Systems

Antagonism between salicylic acid (SA) and JA signaling pathways has been widely reported in plants (Farmer et al. 2003; Spoel et al. 2003; Li et al. 2004; Brodersen et al. 2006; Mao et al. 2007; Koornneef and Pieterse 2008; Zander et al. 2010; El Rahman et al. 2012; Pieterse et al. 2012; Robert-Seilaniantz et al. 2011; Zander et al. 2012; Gimenez-Ibanez and Solano 2013; Van der Does et al. 2013). Antagonistic effect between SA and JA signaling has been reported in tobacco in inducing PR protein genes. The expression of acidic *PR1*, *PR2*, and *PR3* genes was upregulated by SA treatment, and it was suppressed in the presence of JA in a dose-dependent manner, while *PR2*, *PR5*, and *PR6* genes were upregulated by JA and suppressed by SA (Niki et al. 1998). The tobacco basic PR protein gene *NtPRB1b* responds positively to JA and ET signaling systems, but negatively to SA (Niki et al. 1998). JA signaling antagonizes SA signaling system in *Arabidopsis thaliana* (Kloek et al. 2001). The *Arabidopsis* mutants, which accumulate high levels of SA, show repression of JA-mediated pathway (Kachroo et al. 2003a, b). SA promotes disease development caused by necrotrophic pathogens by suppressing JA signaling system (El Rahman et al. 2012).

2.19.2 SA May Block JA Biosynthesis

Transgenic *Arabidopsis* plants expressing NahG, which were unable to accumulate SA, produced 25-fold higher levels of JA in response to infection by *Pseudomonas syringae* pv. *tomato* DC3000 (Spoel et al. 2003). The results

suggest that SA may suppress JA biosynthesis. SA blocks JA biosynthesis in tomato leaves (Peňa-Cortés et al. 1993). SA produced during pathogen infection plays an important role in the suppression of JA biosynthesis. Lipoxygenase (LOX) is the key enzyme in the JA biosynthesis pathway. Pathogen-induced SA suppresses *LOX2* gene encoding LOX2 in *Arabidopsis* (Spoel et al. 2003). Compared with wild-type plants, transgenic NahG plants showed enhanced expression of *LOX2* and accordingly synthesized 25-fold higher levels of JA during pathogen infection (Spoel et al. 2003). Allene oxide synthase (AOS) is another enzyme involved in JA biosynthesis (Laudert and Weiler 1998). Accumulation of allene oxide synthase transcripts in flax leaves is inhibited by salicylic acid (Harms et al. 1998). SA has been suggested to suppress the expression of the JA biosynthetic enzymes lipoxygenase (Spoel et al. 2003) and allene oxide synthase (AOS) (Harms et al. 1998). Leon-Reyes et al. (2010b) showed that the JA biosynthesis genes *LOX2* encoding lipoxygenase, *AOS* encoding allene oxide synthase, *AOC2* encoding allene oxide cyclase, and *OPR3* encoding 12-oxo-phytodienoate reductase were all repressed by SA in *Arabidopsis*. Collectively these studies suggest that SA may suppress the biosynthesis of JA, resulting in the suppression of JA signaling system (Fig. 2.20).

2.19.3 SA May Suppress JA-Responsive Gene Expression

SA strongly antagonized the JA signaling pathway, resulting in the downregulation of a large set of JA-responsive genes, including the marker genes *PDF1.2* and *VSP2* in *Arabidopsis thaliana* (Leon-Reyes et al. 2010b). In the *Arabidopsis aos/dde2* mutant, the expression of the JA biosynthesis enzyme allene oxide synthase (AOS) was completely blocked. Mutant *aos/dde2* plants did not express the JA-responsive marker genes *PDF1.2* or *VSP2* in response to infection with the necrotrophic fungus *Alternaria brassicicola*. Bypassing JA biosynthesis by exogenous application of methyl jasmonate (MeJA) rescued this JA-responsive phenotype in *aos/dde2*. Application of SA suppressed MeJA-induced *PDF1.2* expression to the same level in the *aos/dde2* mutant as in wild-type plants (Leon-Reyes et al. 2010b). The results suggest that SA-mediated suppression of JA-responsive gene expression is targeted at a position downstream of the JA biosynthetic pathway.

SA has been shown to suppress JA signaling downstream of the COI1–JAZ receptor complex (Van der Does et al. 2013). SA is a potent suppressor of JA-inducible gene expression (Doares et al. 1995; Harms et al. 1998; Gupta et al. 2000). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by JA (Doares et al. 1995). The expression of the JA-responsive genes *VSP* and *PDF1.2* was enhanced strongly in the transgenic SA-degrading *NahG* gene expressing *Arabidopsis* plants (Spoel et al. 2003). The results suggest that in wild-type plants JA signaling is inhibited by SA that accumulates during pathogen infection.

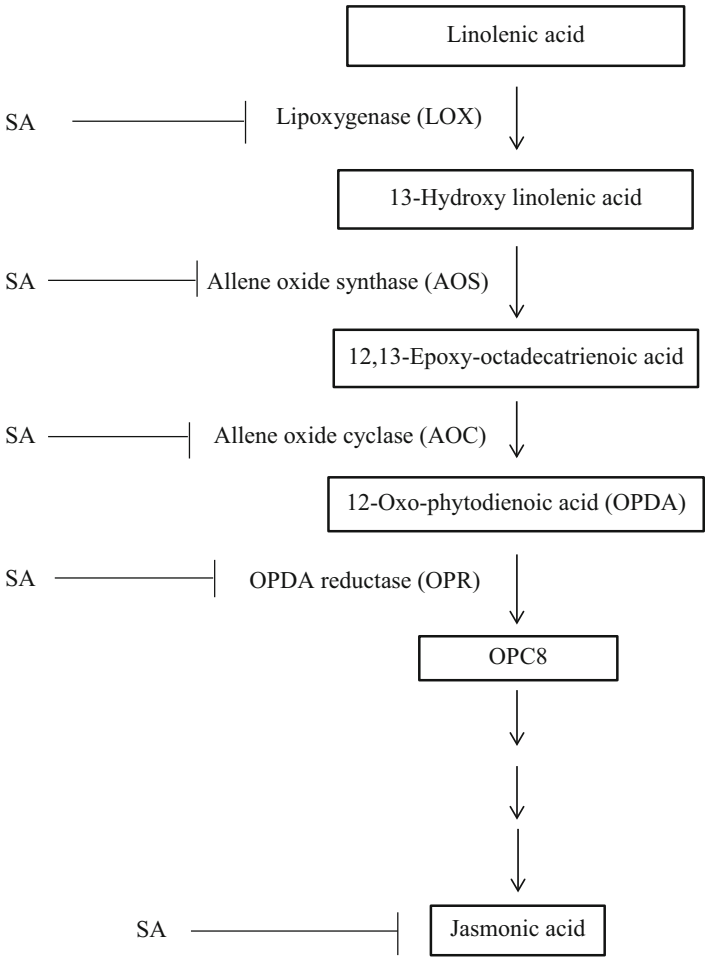

Fig. 2.20 Inhibition of JA biosynthesis enzymes by SA (Adapted from Spoel et al. 2003; Leon-Reyes et al. 2010b)

2.19.4 NPR1 in the Cytosol Modulates Crosstalk Between SA and JA Signaling Systems

NPR1 (non-expresser of PR gene1), the transcription cofactor, has been shown to be involved in the suppressive action of SA against JA signaling (Spoel et al. 2003). NPR1 forms a high-molecular-weight oligomeric complex in the cytosol. SA accumulation causes changes in cellular redox potential, and it leads to the formation of NPR1 monomers that are translocated into the nucleus. However, the NPR1 homomeric complex remains in the cytoplasm (Mou et al. 2003). SA-induced suppression of the

JA response is controlled by the function of NPR1 in the cytosol (Spoel et al. 2003; Koornneef and Pieterse 2008). NPR1 is translocated to the nucleus upon activation by SA, where it facilitates the activation of SA-responsive PR genes. In the cytosol, the remaining SA-activated NPR1 pool may be involved in the suppression of JA-responsive gene expression, either by facilitating the delivery of negative regulators of JA-responsive genes to the nucleus or by inhibiting positive regulators of JA-responsive gene expression (Spoel et al. 2003).

The NPR1 5'UTR contains several W-boxes, which are binding sites for WRKY transcription factors (Wasternack 2007). Some of the NPR1-activated transcription factors are involved in suppressing the activation of JA-responsive genes. The transcription factor WRKY62 is synergistically induced by MeJA and SA (Mao et al. 2007). MeJA inducibility of *WRKY62* is mediated by NPR1. Analysis of *wrky62* mutant and WRKY62-overexpressing plants showed that *WRKY62* suppresses JA-responsive gene expression (Mao et al. 2007). *WRKY62* may function downstream of cytosolic NPR1 and regulate SA-mediated inhibition of JA signaling (Mao et al. 2007).

The *Arabidopsis* transcription factor, WRKY70, is involved in crosstalk between SA and JA signaling pathways. WRKY70 is a common transcription factor in SA- and JA-mediated signaling pathways. It acts downstream of SA and *NPR1* (Knoth et al. 2007) and inhibits JA-responsive genes (Li et al. 2004). JA negatively regulates *WRKY70* transcript levels, whereas SA regulates *WRKY70* transcription positively (Li et al. 2004). *WRKY70* mRNA levels are enhanced in a *coi1* background, suggesting that JA represses its expression (Li et al. 2004). WRKY70 acts as a positive regulator of SA-responsive genes, while it acts as negative regulator of the expression of JA-responsive genes downstream of JA (Li et al. 2004, 2006; Journot-Catalino et al. 2006). WRKY70 activates SA-inducible *PR* genes and represses the JA-inducible gene *PDF2.1* (Fig. 2.21; Li et al. 2006, 2007). WRKY70-mediated suppression of most of the JA-responsive genes appeared to require functional NPR1 (Li et al. 2006).

Transgenic *Arabidopsis* plants overexpressing *WRKY70* have been developed. These plants showed high resistance to the biotrophic powdery mildew pathogen *Golovinomyces cichoracearum* (=*Erysiphe cichoracearum*), but showed enhanced susceptibility to the necrotrophic fungal pathogen *Alternaria brassicicola* (Li et al. 2006). It is known that SA induces resistance against biotrophic pathogens, whereas JA confers resistance against necrotrophic pathogens. The results suggest that WRKY70 positively regulates SA signaling system while it negatively regulates JA signaling system.

WRKY11 and WRKY17 of *Arabidopsis* have also been implicated in SA/JA crosstalk. In a double mutant in which *WRKY11* and *WRKY17* were knocked out, transcripts of SA-responsive genes accumulated to higher levels, whereas those of JA-responsive genes were significantly lower. Expression of *WRKY70* was up-regulated in this double mutant, suggesting that WRKY11 and WRKY17 function as negative regulators of WRKY70 (Journot-Catalino et al. 2006).

The transcription factor OsWRKY13 activates salicylic acid synthesis-related genes and SA-responsive genes, while it suppresses JA synthesis-related genes and

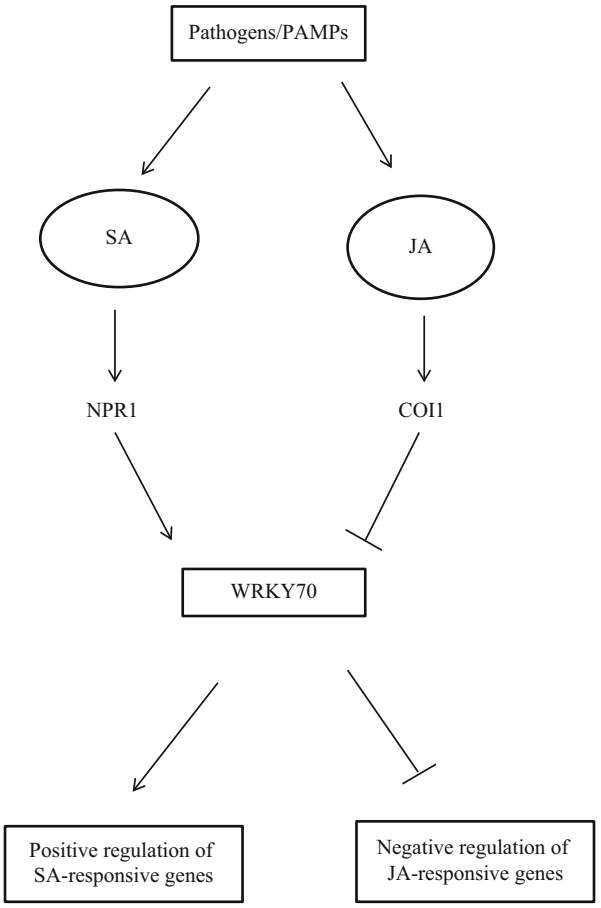

Fig. 2.21 Role of WRKY70 in crosstalk between SA and JA signaling pathways (Adapted from Li et al. 2004, 2006, 2007)

JA-responsive genes (Qiu et al. 2007). It was observed that OsWRKY13 bound to the promoters of its own and at least three other genes in SA- and JA-dependent signaling pathways (Qiu et al. 2007).

2.19.5 Role of Glutaredoxin and TGA Transcription Factors in the SA–JA Crosstalk

SA/JA crosstalk may also be redox regulated. The antagonistic effect of SA on JA-responsive gene transcription has been shown to be linked to SA-induced changes in the cellular redox potential (Koornneef and Pieterse 2008). A glutaredoxin GRX480 has been reported to be involved in SA/JA crosstalk (Ndamukong et al. 2007). Glutaredoxins mediate redox regulation of proteins by their capacity to catalyze disulfide transitions. Basic/leucine zipper-type transcription factors of the TGA family

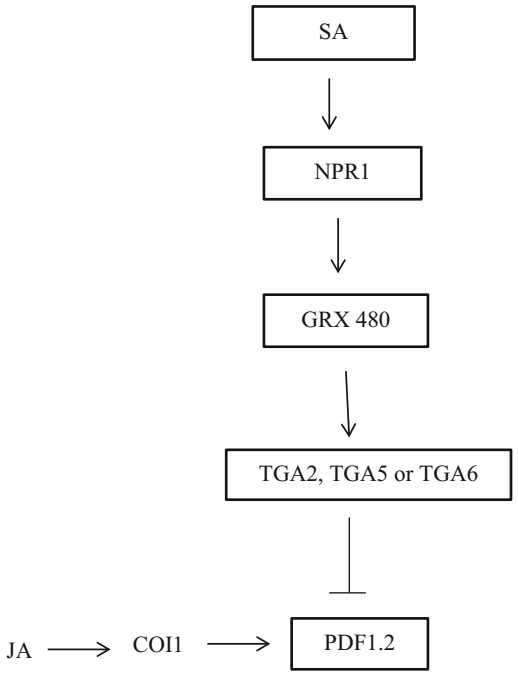

Fig. 2.22 SA-inducible glutaredoxin (GRX480) interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription (Adapted from Ndamukong et al. 2007)

interact with GRX480. Expression of GRX480 was found to be inducible by SA and dependent on NPR1. Transgenic *Arabidopsis* plants expressing *GRX480* showed near wild-type expression of SA-inducible responses, while transcription of JA-dependent defensing gene *PDF1.2* was suppressed in the transgenic plants. Suppression of JA-inducible gene depended on the presence of TGA factors. The interaction between GRX480 and TGA transcription factors was essential for the GRX480-dependent crosstalk. It is suggested that SA-activated NPR1 induces GRX480, which in turn interacts with TGA transcription factors (TGA2, TGA5, or TGA6) to suppress JA-responsive gene transcription (Fig. 2.22; Ndamukong et al. 2007).

2.19.6 Role of MAP Kinase 4 (MPK4) in SA and JA Crosstalk

A MAP kinase, MPK4 has been shown to be involved in SA and JA crosstalk. It acts as a negative regulator of SA signaling and as a positive regulator of JA signaling in *Arabidopsis* (Petersen et al. 2000). Inactivation of MPK4 in the mutant *mpk4 Arabidopsis* plants resulted in increased expression of SA-responsive genes and suppression of JA-responsive genes (Petersen et al. 2000). The target of MPK4 is its substrate MAP KINASE4 SUBSTRATE 1 (MKS1), and phosphorylation of MKS1 by MPK4 represses SA signaling. MSK1 interacts with the WRKY

transcription factors WRKY25 and WRKY33, and both of them can be phosphorylated by MPK4 (Andreasson et al. 2005). Overexpression of both WRKY25 and WRKY33 results in repression of SA signaling responses, suggesting that these transcription factors, after activation by phosphorylation by the action of MPK4, suppress SA signaling system (Zheng et al. 2006, 2007). By contrast, *wrky33* mutant plants showed reduced expression of JA-responsive genes, suggesting that WRKY33 after phosphorylation by MPK4 activates JA signaling system (Zheng et al. 2006). These studies reveal that MPK4 is involved in crosstalk between SA and JA signaling systems.

EDS1 and PAD4 are defense regulators, and they affect SA/JA signal antagonism as activators of SA and as repressors of JA defenses (Wiermer et al. 2005; Brodersen et al. 2006). EDS1 and PAD4 regulate accumulation of SA in *Arabidopsis* (Bartsch et al. 2006). The *eds1* and *pad4* mutant *Arabidopsis* plants were highly susceptible to the bacterial pathogen *Pseudomonas syringae*. Constitutive overexpression of *EDS1* and *PAD4* enhanced resistance to the bacterial pathogen (Xing and Chen 2006). Enhanced resistance to the bacterial pathogen in transgenic *EDS1*- and *PAD4*-overexpressing lines was associated with a quicker and strong induction of SA-regulated defense gene *PR1* after infection with the pathogen. By contrast, *eds1* and *pad4* mutants were tolerant to the fungal pathogen *Botrytis cinerea*. Constitutive overexpression of *EDS1* and *PAD4* restored the susceptibility of the mutants to *Botrytis cinerea*. The negative role of EDS1 and PAD4 in plant response to the necrotrophic fungal pathogen *B. cinerea* correlated with their negative effects on induction of jasmonate-regulated *PDF1.2* gene expression (Xing and Chen 2006). The function of EDS1 and PAD4 in the antagonistic interaction between SA and JA depends on a MAP kinase, MPK4 in *Arabidopsis* (Brodersen et al. 2006).

Both SA-repressing and JA-activating functions of EDS1 and PAD4 appear to depend on MPK4 kinase activity in *Arabidopsis*. Mutations in *EDS1* and *PAD4* genes suppress derepression of the SA pathway and suppress the JA pathway in *mpk4* mutant plants (Brodersen et al. 2006). These results suggest that EDS1/PAD4 affect SA/JA signal antagonism as activators of SA and as repressors of JA defenses and MPK4 negatively regulates both of these functions.

2.19.7 SA May Suppress JA Signaling by Targeting GCC-Box Motifs in JA-Responsive Promoters

It has been now well demonstrated that SA suppresses JA pathway. The suppression of the JA pathway by SA may function downstream of the E3 ubiquitin ligase Skp–Cullin–F-box complex SCF^{COI1}, which targets ZIM-domain transcriptional repressor proteins (JAZs) for proteasome-mediated degradation (Chini et al. 2007; Thines et al. 2007; Pauwels and Goossens 2011; Van der Does et al. 2013). SA does not affect the stability or JA-induced degradation of JAZs. It was observed that the 1-kb promoter regions of JA-responsive genes that were suppressed by SA were significantly enriched in the JA-responsive

GCC-box motifs. SA may suppress JA signaling by targeting GCC-box motifs in JA-responsive promoters via a negative effect on the accumulation of the APETALA2/Ethylene response factor (AP2/ERF)-type transcriptional activator ORA59 (Octadecanoid-responsive Arabidopsis 59). The GCC-box motif is overrepresented in JA-responsive promoters that are suppressed by SA, and this promoter was found to be sufficient for SA-mediated suppression of JA-induced gene expression (Van der Does et al. 2013). SA reduced the accumulation of the GCC-box binding transcription factor ORA59 but not that of ERF1. Collectively these studies show that the SA pathway inhibits JA signaling by targeting GCC-box motifs in JA-responsive promoters (Gimenez-Ibanez and Solano 2013; Van der Does et al. 2013).

2.19.8 JA May Inhibit SA Signaling

It is widely reported that SA inhibits JA signaling and there are also reports indicating that JA also may inhibit SA signaling. In tobacco, MeJA induced the transcription of a glucosyltransferase, a homologue of UDP-glucose/salicylic acid glucosyltransferase in tobacco (Lee and Raskin 1999) and *Arabidopsis* (Sasaki et al. 2001). It is suggested that MeJA inhibits salicylic acid signaling through the induction of the glucosyltransferase, because the sugar conjugate of salicylic acid is inactive (Sasaki et al. 2001).

2.19.9 Synergism Between SA and JA Signaling Pathways

Synergism between SA and JA pathways has also been reported (Mur et al. 2006). A transient synergistic enhancement in the expression of genes associated with either JA (*PDF1.2* [defensin] and *Thi1.2* [thionin]) or SA (*PR1* [PR1a in tobacco]) signaling was observed when both jasmonic acid and salicylic acid were applied at low concentrations. However, antagonism was observed at more prolonged treatment times or at higher concentrations. Similar results were also observed when adding the jasmonate precursor α-linolenic acid with salicylic acid (Mur et al. 2006). These results suggest that the outcomes of JA–SA interactions depend on the relative concentration of JA and SA.

Synergism between JA/ET pathways and SA pathway in inducing resistance against *Plectosphaerella cucumerina* in *Arabidopsis* has been reported (Berrocal-Lobo et al. 2002). Components of the ET and JA pathways work in combination with SA-mediated resistance response in the defense of *Arabidopsis* against *P. syringae* and *Hyaloperonospora parasitica* (Clarke et al. 2000).

2.20 Crosstalk Between SA and ET Signaling Systems

Synergism between SA and ET signaling systems in rice has been reported (Mitsuhara et al. 2008). SA and ET work synergistically and induce resistance against *Cucumber mosaic virus* in *Arabidopsis* (Takahashi et al. 2002). A positive cooperation between ET and SA pathways was observed in *Arabidopsis* plants in response to *Plectosphaerella cucumerina* (Berrocal-Lobo et al. 2002). SA and ET/JA signaling pathways synergistically induced the expression of *PDF1.2* in *Arabidopsis* (Devadas et al. 2002). A negative crosstalk between SA and ET has also been shown in *Arabidopsis* in inducing resistance to *Pseudomonas syringae* pv. *tomato* (Berrocal-Lobo et al. 2002).

2.21 Crosstalk Between SA and ABA Signaling Systems

Abscisic acid suppresses salicylic acid-dependent signaling mechanisms (Audenaert et al. 2002). ABA suppresses SA accumulation in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato* (Mohr and Cahill 2007). The ABA-deficient mutant of tomato had greater SA-mediated responses, suggesting the antagonistic interaction between ABA and SA signaling systems (Thaler and Bostock 2004). ABA reduced SA defense gene expression in *Arabidopsis* (Adie et al. 2007). ABA inhibits the accumulation of SA and the expression of SA-mediated defense genes (Mohr and Cahill 2007; Spoel and Dong 2008). ABA suppresses inducible innate immune responses by down-regulating SA biosynthesis and SA-mediated defenses in *Arabidopsis* (de Torres et al. 2009). ABA has been shown to promote susceptibility to the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* by suppressing SA-mediated defenses (Xu et al. 2013). In contrast, ABA signals have been shown to promote SA biosynthesis in *Arabidopsis* (Seo and Park 2010).

2.22 Crosstalk Between SA and Auxin Signaling Systems

SA has been shown to repress auxin signaling pathway and induce resistance against diseases (Wang et al. 2007). The SA analog BTH treatment repressed 21 genes related to auxin signal transduction. It also affected auxin homeostasis by lowering the levels of free auxin (Wang et al. 2007). Auxin may also suppress the induction of SA signaling. In plants overexpressing the auxin receptor *AFB1*, the accumulation of SA or the induction of SA response pathway is impaired after *P. syringae* pv. *tomato* DC3000 challenge inoculation in *Arabidopsis* (Robert-Seilaniantz et al. 2011).

2.23 Negative Regulation of Salicylate-Mediated Immunity by Brassinosteroid Signaling

Brassinosteroid (BR) signaling induced susceptibility to the rice root pathogen *Pythium graminicola* by suppressing the plant innate immunity. BR treatment induced susceptibility, while an inhibitor of BR signaling, brassinazole (BRZ), induced disease resistance activating plant immunity. These results suggest that BRs are negative regulators of innate immunity in rice plants. In contrast, SA signaling triggered immune responses against the pathogen in rice plants. Application of the brassinosteroid brassinolide (BL) negated resistance conferred by the SA synthetic analog benzothiadiazole (BTH), suggesting negative crosstalk between BR and SA signaling pathways. Exogenous application of BL decreased the pathogen-induced strong upregulation of the transcription of the SA regulatory genes *OsNPR1* and *OsWRKY45*. The temporal expression of both the SA regulatory genes was inversely correlated with that of the BR marker genes *OsBLE2*, *OsCPD1*, and *OsRAVL1* (De Vleeschauwer et al. 2012). These studies suggest that BR-mediated suppression of SA defenses occurs downstream of SA biosynthesis, but upstream of NPR1 and OsWRKY45 in the SA signaling pathway (De Vleeschauwer et al. 2012).

2.24 SA Signaling System May Induce Resistance Against a Wide Range of Pathogens

2.24.1 SA Signaling System Is Involved in Conferring Fungal and Oomycete Disease Resistance

SA signaling induces resistance against a wide range of pathogens. It is highly effective against biotrophic and hemibiotrophic fungal and oomycete pathogens and bacterial and viral pathogens. The SA analog benzothiadiazole (BTH), which activates SA signaling downstream of SA accumulation, induces resistance against the biotrophic rust fungal pathogen *Puccinia helianthi* in sunflower plants (Amzalek and Cohen 2007). SA induces resistance against the biotrophic powdery mildew pathogen *Erysiphe orontii* in *A. thaliana* (Thomma et al. 2001). It also induces resistance against the powdery mildew pathogen *Oidium* sp. in tobacco (Nakashita et al. 2002). SA signaling induces resistance against the biotrophic downy mildew oomycete pathogen *Hyaloperonospora parasitica* in *Arabidopsis* (Genger et al. 2008). Probenazole, which activates SA signaling system at SA accumulation stage as well as at NPR1 stage, triggers resistance against *H. parasitica* in *Arabidopsis* (Yoshioka et al. 2001).

SA signaling system is involved in triggering resistance against hemibiotrophic rice blast fungal pathogen *Magnaporthe oryzae* by inducing various defense genes

(Oostendorp et al. 2001). BTH enhanced resistance against the fungal pathogen *Leptosphaeria maculans* in *Brassica napus* plants. It triggered SAR, which was dependent on SA signaling system (Potlakayala et al. 2007). BTH induced resistance against the anthracnose pathogens *Colletotrichum destructivum* in cowpea seedlings (Latunde-Dada and Lucas 2001) and *Colletotrichum orbiculare* in cucumber (Cools and Ishii 2002; Deepak et al. 2006). The SA analog BTH treatment triggered accumulation of SA-inducible PR proteins and conferred resistance against the hemibiotrophic oomycete pathogen *Phytophthora palmivora* in papaya (Zhu et al. 2003).

SA signaling is not effective against the control of necrotrophic pathogens. It confers enhanced susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* (Kachroo et al. 2001, 2003a, b; Nandi et al. 2003, 2005; Genger et al. 2008). SA has been shown to promote disease development caused by *B. cinerea* through NPR1, by suppressing the JA-dependent defense genes (El Rahman et al. 2012). SA signaling pathway contributes to disease development caused by another necrotrophic pathogen *Alternaria solani* in tomato (El Rahman et al. 2012).

2.24.2 SA Signaling System Is Involved in Conferring Bacterial Disease Resistance

SA signaling system induces resistance against various bacterial pathogens. The SA analog BTH induced resistance against the fire blight pathogen *Erwinia amylovora* in apple leaf tissues by modulating redox systems (Sklodowska et al. 2010). SA-mediated defense responses promote resistance against the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Xu et al. 2013). The *NPR1* gene is a key regulator of SA-mediated signaling system and acts downstream of SA signal (Chern et al. 2008; Mukherjee et al. 2010). Transgenic rice plants overexpressing *NH1*, a rice homolog of *Arabidopsis NPR1* involved in SA signaling, show high levels of resistance to *X. oryzae* pv. *oryzae* (Chern et al. 2005). BTH enhanced resistance against the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* in *Brassica napus* plants. It triggered SAR, which was dependent on SA signaling system (Potlakayala et al. 2007).

MYB96 transcription factor-mediated abscisic acid (ABA) signals promote SA biosynthesis, and the activated SA signaling system triggers resistance against the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis* (Seo and Park 2010). SA signaling system induces resistance against *Pseudomonas syringae* pv. *tomato* in *Arabidopsis* (Thaler and Bostock 2004; Genger et al. 2008; Camañes et al. 2012). Transgenic *Arabidopsis* plants overexpressing *AtRBP-DR1*, the gene encoding RNA-binding protein, were developed, and these transgenic plants showed high accumulation of SA (Qi et al. 2010). Overexpression of *AtRBP-DR1* led to enhanced resistance to *P. syringae* pv. *tomato* DC3000, suggesting that SA induces resistance against the bacterial pathogen (Qi et al. 2010).

The *RAR1* and *SGT1b* genes are required for SA accumulation in *Arabidopsis*, and both are required in a genetically additive manner for induction of disease resistance (Zhou et al. 2008). Overexpression of *OsRAR1* and *OsSGT1* in rice significantly induced basal resistance to the bacterial pathogen *X. oryzae* pv. *oryzae* (Wang et al. 2008). Silencing of the regulator genes *RAR1* and *SGT1-2* involved in SA-mediated defense resulted in susceptibility to *Pseudomonas syringae* in soybean (Fu et al. 2009). These results suggest that SA signaling system is involved in inducing resistance against bacterial pathogens.

The genes encoding WRKY transcription factors functioning in SA signaling system also induce resistance against bacterial pathogens (Miao and Zentgraf 2007; Xing et al. 2008). SA induces the interaction of the transcription factors WRKY38 and WRKY62 with Histone Deacetylase19 (HDA19) and fine-tunes plant basal defense responses against *P. syringae* in *Arabidopsis* (Kim et al. 2008). Constitutive expression of the SA-inducible transcription factor *WRKY18* in *Arabidopsis* enhanced resistance to *P. syringae* (Xu et al. 2006). Overexpression of *OsWRKY13* activates SA synthesis-related genes and SA-responsive genes and enhances resistance to rice bacterial blight (Qiu et al. 2007).

SA signaling system may also induce susceptibility against bacterial pathogens. A rice gene *NRR* interacts with *NPR1* in the SA signaling pathway and suppresses the induction of defense genes, making the plants susceptible to pathogens (Chern et al. 2008). Overexpression of the transcription factors *WRKY38* and *WRKY62* involved in SA signaling system in *Arabidopsis* confers susceptibility to *P. syringae* (Kim et al. 2007). Another pathogen-induced, SA-dependent transcription factor WRKY7 is a negative regulator of host defense. *WRKY7*-overexpressing *Arabidopsis* plants supported more growth of *P. syringae* and developed more severe symptoms than wild-type plants (Kim et al. 2006). Overexpression of the transcription factor *WRKY48*, which is a negative regulator of SA-induced defense responses, suppresses basal resistance to *P. syringae* and makes the *Arabidopsis* plants susceptible to the pathogen (Xing et al. 2008).

CMPA (3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid) protects rice from infection by the bacterial blight pathogen *X. oryzae* pv. *oryzae* (Nakashita et al. 2003; Nishioka et al. 2005). CMPA acts downstream of SA accumulation and acts in the SA signaling pathway between SA production and NPR1 activity (Yasuda 2007). It enhances resistance of tobacco to *Pseudomonas syringae* pv. *tabaci* (Yasuda et al. 2003).

Probenazole induces resistance against rice bacterial blight (*X. oryzae* pv. *oryzae*) by inducing various defense genes (Oostendorp et al. 2001). It induces the expression of S-adenosyl-L-methionine (SAM): salicylic acid carboxyl methyltransferase (SAMT) in *Atropa belladonna*. The enzyme is responsible for the conversion of SA to methyl salicylate (MeSA) (Kwon et al. 2009). MeSA is involved in triggering SA-induced systemic acquired resistance. Tobacco plants treated with probenazole showed increased levels of free and total SA (Midoh and Iwata 1996; Nakashita et al. 2002). The results suggest that probenazole intervenes in SA signaling system at SA accumulation stage as well as at NPR1 stage to trigger resistance against *X. oryzae* pv. *oryzae* (Yoshioka et al. 2001).

Activation or suppression of SA signaling system may induce resistance or susceptibility against the crown gall disease caused by *Agrobacterium tumefaciens* in *Nicotiana benthamiana* (Anand et al. 2008). SA specifically induces resistance against *A. tumefaciens*. SA decreases virulence and attachment of the bacteria to plant cells. It inhibits induction of virulence (*vir*) genes and the *repABC* operon. Plant genes involved in SA biosynthesis and signaling are important determinants for *A. tumefaciens* infectivity on plants. Silencing of *ICS* (isochorismate synthase, the enzyme involved in the biosynthesis of SA), *NPR1* (the regulator of SA signaling), and *SABP2* (SA-binding protein 2) in *N. benthamiana* enhanced *Agrobacterium* infection (Anand et al. 2008). These results suggest that induction of SA signaling induces resistance, while suppression of it induces susceptibility (Anand et al. 2008).

2.24.3 SA Signaling System Is Involved in Conferring Virus Disease Resistance

SA signaling system triggers defense responses against various virus diseases. The SA analog BTH is involved in triggering SA signaling system downstream of SA biosynthesis. It is effective in controlling various virus diseases, such as *Tobacco mosaic virus* (TMV) in tobacco (Friedrich et al. 1996; Waller et al. 2006), *Cucumber mosaic virus* (CMV) in tomato (Anfoka 2000), and *Tomato spotted wilt virus* (TSWV) in tobacco (Mandal et al. 2008). BTH was found to be effective in controlling TSWV in field-grown tomato (Momol et al. 2004).

SA signaling system confers resistance against *Cauliflower mosaic virus* in *Arabidopsis* (Love et al. 2005) Compromising early SA accumulation increases viral dispersal during lesion establishment in TMV-infected tobacco (Mur et al. 1997). Induced resistance against *Turnip crinkle virus* in *Arabidopsis* also is SA dependent (Kachroo et al. 2000).

SA appears to be a key signal molecule in activating defense responses in potato. The wild-type potato plants showed resistance to *Potato virus X* (PVX) avirulent strain ROTH1. SA-deficient transgenic potato plant lines overexpressing the bacterial enzyme salicylate hydroxylase (NahG), which degrades SA, were developed. These transgenic plants showed a decreased disease resistance response to infection by the virus strain (Sänchez et al. 2010). The results suggest that SA content determines disease resistance or susceptibility to the virus disease in potato.

SA signaling system may also be involved in inducing susceptibility to virus diseases. SA signaling system has been shown to enhance virulence of *Clover yellow vein virus* (ClYVV) in susceptible pea cultivars. The virus induced the SA-responsive chitinase gene (*SA-CHI*) in the susceptible pea cultivar. Application of SA or its analog BTH enhanced virulence of a mutant of the virus with reduced virulence. BTH enhanced disease symptoms induced by the wild-type virulent strain of ClYVV (Atsumi et al. 2009). These results suggest that the SA signaling may be associated with susceptibility in pea–*Clover yellow vein virus* interaction.

2.25 Pathogens May Suppress SA Signaling System to Cause Disease

2.25.1 Pathogens May Secrete Effectors to Suppress SA Signaling System

It is widely reported that SA signaling system triggers defense responses against a wide range of pathogens. Virulent pathogens have to overcome the immune responses induced by SA signaling system to cause disease. Pathogens produce effectors to suppress the plant immune responses. The effector proteins target basic innate immunity in plants (Metz et al. 2005; Block et al. 2008; Bartetzko et al. 2009; Boller and He 2009; de Jonge and Thomma 2009; de Jonge et al. 2010; Khang et al. 2010; Kwon 2010; Song and Yang 2010; Szczesny et al. 2010; Valent and Khang 2010; Block and Alfano 2011; Thomma et al. 2011; Wu et al. 2011).

Several *Pseudomonas syringae* strains use a specialized type III secretion system to deliver effector proteins into host cells to subvert host defense mechanisms, thereby promoting pathogenesis (Hauck et al. 2003; DebRoy et al. 2004; Jelenska et al. 2007; Göhre et al. 2008). *P. syringae* pv. *tomato* effector AvrPtoB (now called $HopAB2_{PtoDC3000}$) suppresses basal defense in *Arabidopsis* (de Torres et al. 2006). The effectors HopPtoM and AvrE of *P. syringae* pv. *tomato* suppress the SA-dependent basal immunity in *Arabidopsis* (DebRoy et al. 2004). Effectors of HopPtoM/AvrE family appear to be widespread in plant-pathogenic bacteria, and they appear to target the defense signaling pathways (DebRoy et al. 2004). The effector Hopl1 (previously named HopPmal) isolated from *P. syringae* pv. *maculicola* strain PmaES4326 suppresses SA accumulation in plant cells (Jelenska et al. 2007). Hopl1 expression in *Arabidopsis* plant cells resulted in a 60 % decrease in the level of SA-inducible *PR1* gene transcript and approximately 50 % lower free and total SA levels. It suggests that Hopl1 suppresses SA accumulation and SA-dependent defenses (Jelenska et al. 2007).

2.25.2 Pathogen Produces Toxin and Suppresses SA Signaling System to Promote Disease Development

The bacterial pathogen *Pseudomonas syringae* produces coronatine that mimics jasmonic acid-isoleucine and promotes opening of bacterial entry, bacterial growth in the apoplast, and systemic susceptibility (Zheng et al. 2012). Coronatine was found to activate three homologous NAC transcription factor (TF) genes through direct activity of the transcription factor MYC2. The activated NAC TFs repress the *isochorismate synthase 1* (ICS1) gene, which is involved in SA biosynthesis.

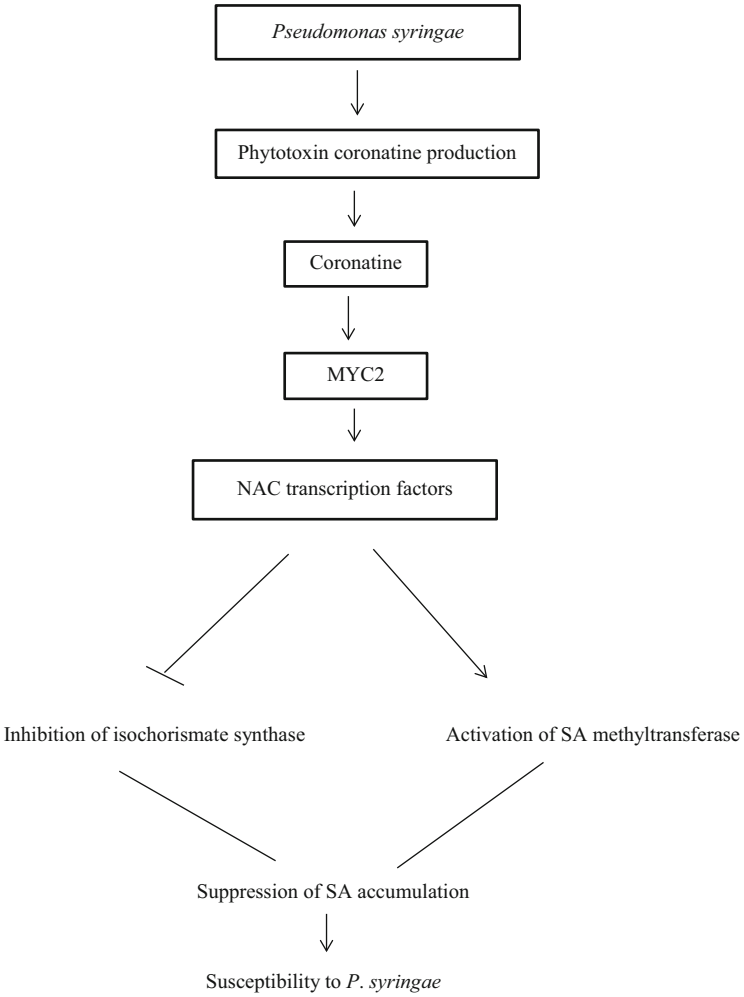

Fig. 2.23 *Pseudomonas syringae* produces phytotoxin coronatine and suppresses SA signaling system to cause disease (Adapted from Zheng et al. 2012)

These TFs also activate expression of *BSMT1* gene encoding SA methyltransferase, which converts SA to the inactive volatile methyl SA (MeSA) (Zheng et al. 2012). These results suggest that the toxin produced by the bacterial pathogen promotes systemic susceptibility by suppressing SA signaling, and these studies also suggest that SA signaling is important in conferring resistance against the bacterial pathogen (Fig. 2.23; Zheng et al. 2012).

2.25.3 *Pathogen Manipulates the Antagonistic Effect Between SA and JA Signaling Systems to Promote Disease Development*

SA signaling system is known to induce resistance against the bacterial pathogen *P. syringae* pv. *tomato* in *Arabidopsis* (Brooks et al. 2005; Jelenska et al. 2007). When *Arabidopsis* plants were inoculated with *P. syringae* pv. *tomato*, there was substantial increase in *WRKY33* transcript level (Dong et al. 2003; Zheng et al. 2006). WRKY33 induces JA-mediated signaling system, and the induced JA signaling system reduces the SA-mediated signaling system by its antagonistic function (Zheng et al. 2006; van Verk et al. 2008). It suggests that the pathogen suppresses the SA signaling pathway, which is needed to induce resistance against the pathogen, to cause disease.

Activation of SA-mediated pathway appears to confer resistance to *Pseudomonas syringae* pv. *tomato* DC3000. The pathogen produces the toxin coronatine, which induces JA-signaling pathway while suppressing the SA-dependent pathway (Katsir et al. 2008). It suggests that the pathogen is able to suppress the signaling pathway which may suppress its pathogenesis and diverts the signals for activation of an inefficient pathway resulting in defense response that cannot inhibit its pathogenesis (Spoel et al. 2003, 2007; Uppalapati et al. 2007). The PR1 protein is required for the inhibition of *P. syringae* pv. *tomato* (Laurie-Berry et al. 2006), and the induction of this pathogenesis-related protein is dependent on SA accumulation (van Verk et al. 2008). The toxin produced by the pathogen triggers JA signaling, which suppresses SA-mediated defenses. Thus, it appears that the bacterial pathogen utilizes the toxin to manipulate JA signaling both to suppress SA-mediated responses and to promote symptom development (Brooks et al. 2005; Laurie-Berry et al. 2006; Koornneef and Pieterse 2008).

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