Protease Inhibitors and Stress Tolerance

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ABSTRACT

Plant protease inhibitors (PIs) are extensively studied for their role in defense against pests and pathogens due to their ability to inhibit specific proteases of the intruder. All classes of PIs were found to play important roles in innate host defense mechanisms and are used to develop transgenic plants resistant to pests and pathogens. Recent reports suggest that they have a role in modulating abiotic stress tolerance also. The induction of PIs was observed in response to not only biotic but also abiotic cues suggesting their possible role in abiotic stress responses and in modulating tolerance to various stresses. During biotic stress, pathogen invasion or damage due to herbivory, induces SA/JA-mediated signalling pathways to combat the attack. Abiotic stress induces injury and different conditions like osmotic stress, oxidative stress etc. as long as the causative factor exists in the milieu. Unlike in the biotic stresses, the causative agent in abiotic stress cannot be countered by the plant. Hence, the abiotic stress has to be tolerated by the plant nullifying all its related damages mostly by adjustment through ABA-dependent, and sometimes through an independent pathway. The overexpression of PIs has been shown to enhance abiotic stress tolerance in the transgenic plants suggesting that their role is multidimensional. In this review, we focus on the possible role of PIs in plant growth, and biotic and abiotic stress tolerance.

CONTENTS

INTRODUCTION

Plants have sophisticated mechanisms to protect themselves from changing environmental factors, which adversely influence their growth, survival and reproduction. These include both biotic as well as abiotic factors. The stresses in general occur in multitude and plants induce different pathways under the control of various stress hormones to survive unfavourable situations. Hormones like jasmonic acid (JA), salicylic acid (SA), ethylene (ET) are known to be particularly induced under biotic stress conditions, whereas abscisic acid (ABA) is induced under abiotic stress conditions (Fujita *et al.* 2006; Cao *et al.* 2011). The later studies have shown that all the hormones are involved in stress responses and work synergistically or antagonistically with each other (Fujita *et al.* 2006). The growth hormones viz., cytokinins, auxins, gibberellic acid and brassinosteroids have also been recognized as important players in plant immunity (Mauch-Mani and Mauch 2005; Grant and Jones 2009). As all the stress hormones are induced under biotic

and abiotic stress conditions, their downstream pathways are also induced with possible overlapping and crosstalk (Fujita *et al.* 2006; Abuqamar *et al.* 2009). The overlapping and crosstalk between different pathways would be modulated by common players for different stresses, which can influence multiple pathways. The basic helix-loop-helix (HLH) transcription factor MYC2 is shown to be involved in the ABA-mediated drought stress signaling pathway (Abe *et al.* 2003). It was later identified that MYC2 up-regulates the expression of genes that are involved in JA mediated wounding response and negatively regulates the expression of JA/ET-mediated pathogen defense genes (Anderson *et al.* 2004; Lorenzo *et al.* 2004). RD26, a dehydration-responsive NAC transcription factor is involved in regulation of both biotic and abiotic signaling. Its expression is induced by JA, ABA, hydrogen peroxide (H_2O_2) and pathogen infections, as well as by drought and high salinity (Fujita *et al.* 2004; Zimmermann *et al.* 2004). It protects the plants by inducing the gene products that are involved in the detoxification of reactive oxygen species (ROS), defense,

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or senescence. Fujita *et al.* (2006) also reported that RD26 functions at the convergence point between the pathways for pathogen defense, senescence, and ABA-mediated signaling. There are now many such reports suggesting that the commonly induced products of different pathways play a substantial role in biotic and abiotic stress tolerances (Wu *et al.* 2009; Orsini *et al.* 2010).

The protease inhibitors are well known for their involvement in biotic stress resistance and are induced by wounding and JA (Green and Ryan 1972; Reymond *et al.* 2000; Ryan 2000; Sasaki *et al.* 2001; Ralph *et al.* 2006; Wasternack 2007; Srinivasan *et al.* 2009). The first convincing evidence that PIs are a part of the natural defensive chemicals of plants was the demonstration that wounding of tomato and potato leaves by Colorado potato beetles (*Leptinotarsa decemlineata*) induced a rapid accumulation of PI (I), not only in the damaged leaves, but also in distal, undamaged leaves (Green and Ryan 1972). They are of interest not only as potential sources of resistance against pests and pathogens in transgenic plants, but also as drugs with antiviral and other properties as well as providing markers for studies of plant diversity and evolution (Lawrence and Koundal 2002; Korsinczky *et al.* 2004). Conconi *et al.* (1996) have shown that PIs are induced under abiotic stress condition (UV-C radiation) also and many studies have reported subsequently on the induction of PI under abiotic stress conditions and their related signalling molecules (Pernas *et al.* 2000; Kim *et al.* 2001; Dombrowski 2003; Capiati *et al.* 2006). These studies have suggested the involvement of PIs in abiotic stress tolerance also. Later, Huang *et al.* (2007) for the first time showed by functional characterization that the constitutive expression of PI induced drought stress tolerance in transgenic rice plants. Subsequently, PI expressing transgenic tobacco plants have been reported to exhibit salinity, pH and osmotic stress tolerance (Shan *et al.* 2008; Srinivasan *et al.* 2009).

The involvement of PIs in biotic and abiotic stress tolerance is reported and it has been studied extensively in the direction of biotic stress signalling and resistance. In this review we focus on the possible pathways through which PIs are induced under abiotic stress conditions and their role in enhancing abiotic stress tolerance in its transgenic plants.

BIOTIC STRESS AND INDUCTION OF PROTEASE INHIBITORS

The PIs are said to be induced under biotic stresses primarily in wounding by pests and invasion by pathogens (Pearce *et al.* 1993; Peña-Cortés *et al.* 1995; Cardenas *et al.* 2001). The JA dependent pathway plays a major role in the induction of PIs and other stress hormones have synergistic or antagonistic roles (Fujita *et al.* 2006). The cell damage caused by mastication of pests or by enzymes secreted by pathogens leads to the release of systemin (Pearce *et al.* 1991) and other wound-signaling peptides (Ryan *et al.* 2000). Systemin binds to a cell surface receptor kinase and causes alterations in ion transport (Felix and Boller 1995; Scheer and Ryan 2002). The generation of ROS activates the related mitogen activated protein kinases (MAPKs) and increases the levels of intracellular calcium (Stratmann and Ryan 1997; Orozco-Cardenas and Ryan 1999; Orozco-Cardenas *et al.* 2001). Later, phospholipase A2, which acts on the plant membrane releasing linolenic acid (LA) will be activated (Lee *et al.* 1997; Narvaez Vasquez *et al.* 1999). The synthesis of JA from LA is initiated by lipoxygenases followed by allene oxide synthase, and allene oxide cyclase, which form 12-oxo-phytodienoic acid (OPDA) (Schaller 2001; Walter *et al.* 2010). OPDA is reduced by OPDA reductase to yield 3-oxo-2-(2′[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) (Vick and Zimmerman 1984; Li *et al.* 2005). OPC- 8-CoA is produced from OPC-8 by OPC-8: CoA ligase (Koo *et al.* 2006). JA is derived from OPC-8-CoA following three cycles of β-oxidation by acyl-CoA oxidase and L-3-ketoacyl-CoA thiolase (Sasaki *et al.* 2001; Schaller 2001; Wasternack 2007). The pathway of JA syn-
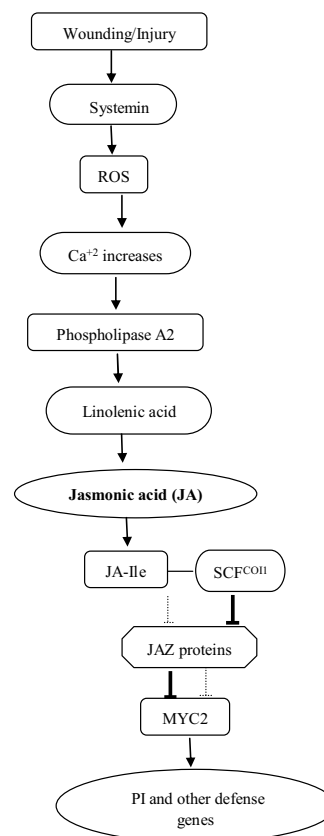

Fig. 1 The schematic representation of induction of PI and other defense genes. The wounding activates systemin, which through ROS and Ca^{2+} ion concentration promotes the release of linolenic acid. The linolenic acid is converted into jasmonic acid through octadecanoid pathway (Wasternack 2007). The JA is converted into its JA-Ile (jasmonic acid and isoleucine) conjugate which complexes with SCF^{COI1} and degrades the JAZ proteins. The MYC2 is now free to interact with the defense genes (Gfeller *et al.* 2006).

thesis has been studied extensively in many plants, but the downstream signalling of JA to induce PIs and other defence proteins was reported in *Arabidopsis* (**Fig. 1**; Gfeller *et al.* 2006; Chung *et al.* 2008).

JA is functionally inactive until it is activated by its conjugation to hydrophobic amino acid, L-isoleucine, mediated by the enzyme JA: amino synthetase, specifically known as jasmonate resistant 1 (Guranowski *et al.* 2007; Walter *et al.* 2010). The absence of JA or its presence at low levels does not induce the expression of defense genes and the JASMONATE ZIM-motif (JAZ) proteins repress the expression of JA-responsive genes by interacting directly with the basic helix-loop-helix transcription factor MYC2 (Gfeller *et al.* 2006; Chung *et al.* 2008), which is a positive regulator of JA responses (Lorenzo *et al.* 2004; Chini *et al.* 2007). CORONATINE-INSENSITIVE1 (COI1) is a Leu-rich repeat/F-box protein that determines the substrate specificity of the SCF-type E3 ubiquitin ligase, SCF^{COI1} (Xu *et al.* 2002; Chung *et al.* 2008). The increase in jasmonoyl isoleucine (JA-Ile) stimulates the binding of JAZs to COI1 and thus, the COI1 complex promotes the ubiquitization of JAZ proteins leading to their proteolysis (Gfeller *et al.* 2006; Chung *et al.* 2008). MYC2 proteins are now free to induce the PIs and other defense genes (Chini *et al.* 2007; Thines *et al.* 2007). The different steps in the pathway have been detailed out in **Fig. 1**.

JA-dependent gene expression is influenced by ET and ABA under the attack of pathogens (Adie *et al.* 2007; Fan *et al.* 2009). The synergistic effect of JA and ET in the activation of defenses against necrotrophs can be explained by the concerted activation of Ethylene Response Factor1 (ERF1), which induces defense gene expression and plant resistance (Berrocal-Lobo *et al.* 2002; Lorenzo *et al.* 2003).

However, in response to wounding, ET and JA antagonize one another and this depends on the balance of activation of ERF1 and MYC2 by both these hormones (Lorenzo *et al.* 2004). ABA synergizes with JA and exhibits a complex antagonistic relationship with SA during disease development. ABA accumulation precedes the onset of JA induction suggesting that ABA may promote JA accumulation and enhance JA action (Fan *et al.* 2009). The role of ET and ABA in the induction of PI under abiotic stress conditions is discussed later.

BIOTIC STRESS RESISTANCE BY OVEREXPRESSION OF PI

The physiological functions of plant PIs are basically the regulation of endogenous proteinases and as a repository of proteins (storage proteins) (Mosolov *et al.* 2001; Shewry 2003). The PI gene expression has been detected in leaves of several species following wounding suggesting their role in protecting plants from insect attack and microbial infection. The possible role of PIs in plant protection was envisaged as early as 1947, when Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Haq *et al.* 2004). The defensive role of PIs is based on their inhibitory activities towards the digestive enzymes of the insect and other pathogen related proteases involved in some vital processes resulting either in a critical shortage of essential amino acids (Hilder *et al.* 1993; Jongsma and Bolter 1997) or interfering with important biochemical or physiological processes of insects and other pathogens, such as the proteolytic activation of enzymes, molting of insects, or replication of viruses (Gutierrez-Campos *et al.* 1999). The activity of PIs is due to their capacity to form stable complexes with target proteases thereby blocking, altering or preventing access to the enzyme active site. Support for a defensive role of plant PIs initially came from studies of insects raised on artificial diets containing PIs and *in vitro* inhibition assays of insect gut proteases with purified PIs from various plant sources. The results of these studies strongly implicate plant PIs in interference with the growth and development of many phytophagous insects (Reeck *et al.* 1997). The correlation between the levels of PIs present in seeds of various cowpea varieties and the resistance to a major insect pest (*Callosobruchus maculatus*) also indicated a protective role for PIs in crops (Gatehouse *et al.* 1979). The direct evidence for the involvement of PIs in the plant defense system has come from studies on transgenic plants. As their role as inhibitors is simply achieved by the activation of single genes, several transgenic plants expressing PIs have been produced in the past two decades and tested for enhanced defensive capacities, with particular efforts against insect pest. Due to these efforts, a more complex scenario about the interaction between insect proteases and plant PIs has started emerging (De Leo *et al.* 2002). A cowpea protease inhibitor (CpTI) was shown for the first time to confer resistance to feeding by the tobacco budworm (*Heliothis virescens*), when the CpTI gene was expressed in transgenic tobacco (Hilder *et al.* 1987). Since then, many insect-resistant transgenic plants have been generated. Plant proteinase inhibitors are also known to confer natural as well as engineered protection against nematode attack (McPherson and Harrison 2001; Atkinson *et al.* 2003). Nematode control with PIs expressed in transgenic tomato (Urwin *et al.* 1995), *Arabidopsis thaliana* (Urwin *et al.* 2000) and rice (Vain *et al.* 1998) has been well demonstrated, and the technology has been patented (Hepher and Atkinson 1992). Transgenic tobacco plants expressing rice cysteine proteinase inhibitor showed enhanced resistance against potyviruses (Gutierrez-Campos *et al.* 1999). Proteinase inhibitors have also been implicated to play a role in the plant's natural defense towards fungal infections (Soares-Costa *et al.* 2002). Trypsin inhibitors (TIs) from buckwheat seeds (Dunaevskii *et al.* 1994) and trypsin and chymotrypsin inhibitors from cabbage foliage (Lorito *et al.* 1994) have been shown to have antifungal activities.

Thus, the PIs are proved to confer biotic stress tolerance effectively upon overexpression.

MODE OF ACTION

It is well known that protease inhibitors act against specific proteases. PIs interact with their target proteases by contact with the active (catalytic) site of the protease resulting in the formation of a stable protease-inhibitor complex that is incapable of enzymatic activity (Norton 1991). The mechanism of action of these PIs has been the subject of intense investigation (Barrett 1986; MacPhalen and James 1987). Knowledge on mechanisms of protease action and their regulation *in vitro* and *in vivo*, in animals, plants, microorganisms and more recently in viruses has been reported and the inhibitory action of PIs over insect proteases was extensively studied (Lawrence and Koundal 2002).

The proteases in insects digest the protein content of the ingested food and these are secreted in the insect midgut depending on the protein content of the food rather than its volume (Baker *et al.* 1984). The secretion of proteases has been attributed to two mechanisms, involving either a direct effect of food components (proteins) on the midgut epithelial cells, or a hormonal effect triggered by food consumption (Applebaum 1985). The digestive proteolytic enzymes in the different orders of commercially important insect pests belong to one of the major classes of proteinases predominantly. Coleopteran and Hemipteran species tend to utilize cysteine proteinases (Murdock *et al.* 1987), while Lepidopteran, Hymenopteran, Orthopteran and Dipteran species mainly use serine proteinases (Ryan 1990; Wolfson and Murdock 1990). PIs inhibit the protease activity of these enzymes thereby reducing the quantity of proteins that can be digested. Coupled with this, the hyperproduction of the digestive enzymes enhance the loss of sulphur containing amino acids (Shulke and Murdock 1983) as a result of which, the insects become weak with stunted growth and ultimately die.

The commonly accepted mechanism of binding of the plant PIs to the insect proteases appears to be similar with all the four classes of inhibitors. The inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^7 to 10^{14} M at neutral pH values), thus effectively blocking the active site. A binding loop on the inhibitor usually "locked" into conformation by a disulphide bond projects from the surface of the molecule and contains a peptide bond (reactive site) cleavable by the enzyme (Terra *et al.* 1996; Walker *et al.* 1998). This peptide bond may be cleaved in the enzyme inhibitor complex, but cleavage does not affect the interaction, so that a hydrolyzed inhibitor molecule is bound similar to an unhydrolyzed one. The inhibitor thus directly mimics a normal substrate for the enzyme, but does not allow the normal enzyme mechanism of peptide bond cleavage to proceed to completion i.e. dissociation of the product (Walker *et al.* 1998).

ROLE OF PROTEASE INHIBITORS IN ABIOTIC STRESS AND ITS TOLERANCE

The common observation that PIs are wound inducible (Peña-Cortés *et al.* 1991; Pearce *et al.* 1993) initially led to the studies focusing on their role in biotic stress (Johnson *et al.* 1989; Klopfenstein *et al.* 1997). The involvement of PI in biotic stress resistance was very well documented explaining their induction, mode of action, insect resistance etc (Green and Ryan 1972; Pearce *et al.* 1991; Ryan *et al.* 2000). Plant hormones like JA and its derivatives, ABA, ET, etc. are reportedly involved in the upregulation of PI as a part of biotic stress response in the plants and these hormones are known to be induced even under abiotic stress conditions (Peña-Cortés *et al.* 1992; Kim *et al.* 2001). Thus, earlier studies using hormones in PI induction have suggested the possibility of their involvement in abiotic stress responses. The later studies under different abiotic stress

conditions confirmed that the PIs are also induced as a response to abiotic stresses.

The expression of PIs in response to drought or salinity stress has been studied by various groups. PIs from chestnut, barley, rice were induced by drought and salinity (Pernas *et al.* 2000; Gaddour *et al.* 2001; Huang *et al.* 2007). Some PIs are induced only through one of the two above mentioned conditions explaining that different PIs are possibly induced under different conditions based on their promoter elements. This also explains that a separate set of PIs respond to abiotic stress conditions with the possibility of overlapping functions.

Salt-induced PI expression was observed at 100 to 300 mM of NaCl up to 24 h in some studies (Dombrowski *et al.* 2003; Srinivasan *et al.* 2009) and progressive drought up to 30 days has also reportedly induced PI expression (Downing *et al.* 1992; Kang *et al.* 2002). Even though salinity induces drought like condition and both the stresses have oxidative stress in common, PI's that are induced by drought are not always induced by salinity and *vice versa*, indicating that the induction of PIs follows independent and may be, interlinked mechanisms. Apart from salinity and drought stresses, other abiotic stresses also induce PIs. *Brassica* PI containing a Kunitz-type PI motif was induced by progressive drought and heat stresses from 30-40°C up to 72 h (Satoh *et al.* 2001). A cystatin was shown to be induced in chestnut by cold shock at 4°C for 4 weeks and heat stress at 32 or 40°C for 3-8 h (Pernas *et al.* 2000). Also, the exposure to UV-C radiation up to 25 h resulted in the synthesis of PI in tomato (Conconi *et al.* 1996). All these studies suggest that the PIs are also involved in abiotic stress responses, though their role in abiotic stress tolerance is yet to be clearly defined.

Many abiotic stress conditions induce the PIs and the plants respond to these stresses through the signalling of stress hormones like JA, ABA, ET (Peña-Cortés *et al.* 1995; Moons *et al.* 1997; Fujita *et al.* 2006). JA is induced in response to abiotic stresses like ultraviolet radiation (Conconi *et al.* 1996), ozone (Rao *et al.* 2000), drought (Fujita *et al.* 2004), etc. It is explained that under these conditions, PIs are induced and JA is also known to be a potent inducer of PIs. The induction of JA under abiotic stress conditions is not clearly explained, but there are some studies that report the crucial players in the process (Fujita *et al.* 2006). Dombrowski (2003) has reported that the tomato mutant (*def-1*) with an impairment in the octadecanoid pathway displayed severe reduction in the accumulation of proteinase inhibitors under salt stress indicating that salt stress-induced accumulation of PIs was jasmonic acid dependent. Prosystemin was not necessary for the induction of PI, but is required for inducing to higher levels. Similarly, it has also been shown that mechanical wounding increases salt stress tolerance through the involvement of systemin and JA (Capiati *et al.* 2006).

Abiotic stress also can induce wounding (Cheong *et al.* 2002) and is associated with other stresses like oxidative, osmotic and dehydration stresses (Boudsocq and Laurière 2005). The stress perception and downstream signalling involve ROS and higher levels of stress enhance ROS production, which results in oxidative stress. Thus, ROS is a common denominator for all biotic and abiotic stresses. The osmotic stress is also induced by the accumulation of sodium chloride, heavy metals and others in the vicinity of plants. The variation in the internal and external concentration of ions in the cell would lead to osmotic stress. Salt stress also decreases the absorption of water by the plant, thereby creating drought stress (Zhu 2001). The initial stress and its latently induced stresses would result in enhanced ROS levels, which would influence different pathways along with the other stress responsive products. There are two major pathways that influence the production of JA under these conditions (**Fig. 1**). In one pathway, ROS can directly influence calcium levels, which induce the production of JA through different Ca^{2+}-dependent protein kinases (CDPKs) (Song *et al.* 2005). In the other pathway, ROS can
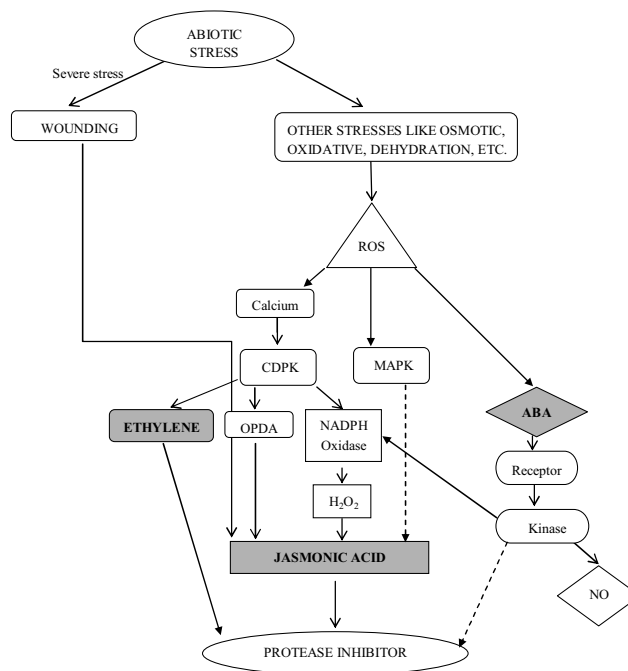

Fig. 2 The possible mechanisms of PI induction under abiotic stress conditions. The abiotic stress induces different stress hormones based on the kind of stress. The JA pathway is known to induce PIs and ET works synergistically in case of wounding to induce PIs (Adams and Turner 2010). ABA known to be the abiotic stress hormone and it induces the production of JA dependent genes through MYC2 transcription factor (Fugita *et al.* 2006; Gfeller *et al.* 2006).

directly induce MAPKs, which, through unknown mechanisms, would stimulate the production of JA (Seo *et al.* 2009; Heinrich *et al.* 2011).

Earlier studies have reported that cytosolic calcium levels increase in plant cells in response to multiple adverse environmental conditions including salinity, drought, cold, pathogen attack, and mechanical wounding (Nurnberger and Scheel 2001; Xiong *et al.* 2002). The enhanced calcium levels activate phosphorylation/dephosphorylation cascades by interacting with sensors such as calmodulin, CDPKs (Harper *et al.* 2004; Bouché *et al.* 2005). The CDPKs are said to be induced in response to several environmental stresses suggesting that these kinases could function as cross-talk mediators between signalling pathways leading to cross tolerance (Sanders *et al.* 2002; Harper *et al.* 2004). CDPK1 in tomato was found to induce salinity tolerance and also involved in the expression of wound induced genes. This was also proposed to be the cross talk node between salt and wounding signalling pathways (Capiati *et al.* 2006; **Fig. 2**). The CDPKs induce the synthesis of JA directly through the octadecanoid pathway (Ludwig *et al.* 2005) thereby leading to the production of PI. The CDPKs also induce NADPH oxidase, which influences the synthesis of H_2O_2 that in turn influences the production of JA (Hu *et al.* 2003; Ludwig *et al.* 2005).

Ethylene (ET) is reported to be induced by ozone, freezing, drought stress etc (Seki *et al.* 2002; Zhao *et al.* 2004). The induced ET is perceived by five different receptors namely ETHYLENE RESPONSE-1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR-1 (ERS1), ERS2, and ETHYLENE INSENSITIVE-4 (EIN4) (Chen *et al.* 2005; Benavente and Alonso 2006; Etheridge *et al.* 2006). The receptors pass the signal downstream to transcription factors like EIN3, which in turn activate other key transcription factors like ETHYLENE RESPONSE FACTOR-1 (ERF1). ERF1 is known to influence the expression of defense genes (PI) and it is also said to be the integration point between the ET and JA pathways (Solano *et al.* 1998; Lorenzo *et al.* 2003). The signalling in wounding induces PI due to the synergistic effect of JA and ethylene, but in abiotic stress

conditions this was not reported (Lorenzo *et al.* 2003; Adams and Turner 2010).

Abiotic stresses like drought, salt, low-temperature, osmotic variation etc induce the accumulation of ABA that plays a crucial role in plant adaptation to abiotic stresses (Finkelstein *et al.* 2002; Fujita *et al.* 2006). The induced ABA persuades changes in Ca^{2+} levels through the regulation of calcium channels by IP3, thus leading to the activation of different kinases (Takahashi *et al.* 2001; Chinnusamy *et al.* 2004). The kinases are further involved in the activation of transcription factors that induce the downstream signalling process. The ABA induced expression often relies on the presence of the *cis*-acting element called ABRE element (ABA-responsive element) in the promoters (Yamaguchi-Shinozaki *et al.* 1990; Shinozaki and Yamaguchi-Shinozaki 2000). The basic leucine zipper transcription factors called AREB bind to ABRE elements in these promoters and induce the stress responsive genes (Hobo *et al.* 1999; Choi *et al.* 2000). Other transcription factors such as the MYC and MYB proteins etc are also involved in ABA-responsive signal transduction and they interact with their corresponding *cis*-acting elements such as DRE/CRT, ABRE and MYCRS/MYBRS, respectively (Fujita *et al.* 2004; Tuteja 2007). In *Arabidopsis*, MYC2 was reported to be involved in the ABA mediated drought stress signalling pathway (Urao *et al.* 2003; Fujita *et al.* 2006). Similarly, the MYC2 was also known to induce defense genes in JA-mediated responses and thus, MYC2 seems to be a nodal point for the crosstalk between ABA and JA pathways (Fujita *et al.* 2006; Gfeller *et al* 2006). The PI could also be expressed by the activity of MYC transcription factors induced by ABA. ABA and JA differentially induce PI and this explains the fact that all PI are not induced in similar conditions.

ABIOTIC STRESS TOLERANCE BY OVER-EXPRESSION OF PI

Under natural abiotic stress conditions, plant tries to induce proteins and non-protein molecules to overcome the effects of the stress. But, the optimum or higher levels of the respective protein(s) is produced constitutively in the transgenics by the overexpression of the relevant gene. The responses will be different in wild type and transgenic plants in any given stress condition. Hence, the above discussed mechanisms would not be appropriate to explain the response of PI transgenics to different abiotic stress conditions.

The ROS normally act as signaling molecules in the cell and changes in the surrounding environment are also transduced into the cell by elevation in the levels of ROS, which above a threshold level, impart toxic effects (Mittler 2002; Miller *et al.* 2008). The intensity of ROS depends on the level of stress. Hence, the higher the stress, the greater the level of ROS. A rise in the level of ROS could be connected to an attempt of the cell to survive against the alarming conditions rather than mere destruction of itself (Mittler *et al.* 2004, 2008). The changing levels of ROS induce stress hormones for further signaling at basal and primary stress induced metabolisms (Kwak *et al.* 2006; Bogatek and Gniazdowska 2007). Hydrolytic enzymes are produced at higher and persistent ROS levels for initiation of cell death phenomenon (Breusegem and Dat 2006; Reape *et al.* 2008). These stress hormones induce enzymatic and non-enzymatic antioxidants to curb the effects of elevated ROS (Mittler *et al.* 2004, 2011). The other stress responsive proteins are also induced for holistic stress tolerance. The hydrolytic enzymes induced at higher levels of ROS will degrade the cell and the major enzymes induced are proteases. The proteases degrade the protein pool of the cell, which includes stress responsive proteins, membrane proteins etc, thus making the cell to succumb to the stress (**Fig. 3**).

The protease inhibitor transgenics have been shown to exhibit enhanced tolerance to abiotic stress conditions like drought, salinity, osmotic variations and pH (Huang *et al.*
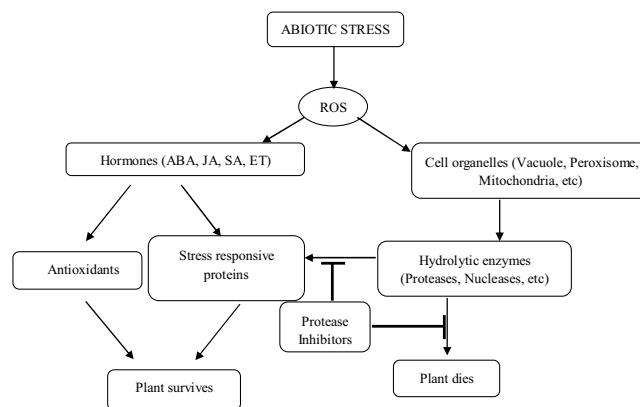

Fig. 3 The possible role of PI in abiotic stress tolerance. The proteases are induced under stress conditions and they degrade the protein pool of the cell (Sahi *et al*. 2006). The PIs are said to inhibit the proteases and thus they increase the life of the proteins, which include stress responsive gene products. These stress responsive proteins will favour the survival of plant under the stress.

2007; Shan *et al.* 2008; Srinivasan *et al.* 2009). The protein degradation and recycling are induced in plants that are subjected to abiotic stress conditions (Ingram and Bartels 1996; El-Maarouf *et al.* 1999; Sahi *et al.* 2006). Protein degradation can be controlled by curbing the proteases and the PIs are the proteins that control this protease activity. The constitutive expression of PIs directly has the advantage of control over the activity of proteases and enhanced protease inhibitory activity is reported in PI transgenics (Huang *et al.* 2007; Srinivasan *et al.* 2009). However, the mechanism by which the PI expressing transgenic plants exhibit enhanced tolerance is not completely understood as inhibition of proteases would not have a direct relation to stress tolerance. The salinity tolerance exhibited by TI or bowman-birk transgenics is reported to be by the inhibition of sodium translocation to other parts of plant from the root system (Shan *et al.* 2008; Srinivasan *et al.* 2009).

The rationale behind this phenomenon to occur in the transgenics is by the regulation of ion channels and discrimination of Na^+/K^+ in the stem region. The Na^+/K^+ discrimination is performed by Kna1 in wheat by sensing the ratio of K/Na (Flowers 2004). Thus, it can be postulated that the PI transgenics inhibit proteases, which degrade proteins that regulate ion channels or Kna1, and related proteins. The pH and osmotic stress tolerance exhibited by PI transgenics could also be due to the control of H^+ and other ion absorption. The complete mechanism of abiotic stress tolerance in these transgenics needs a thorough analysis and apart from ion channel regulation and Kna1, there might be other mechanisms that favor the transgenics.

FUTURE PROSPECTS

The reports of PI involvement in abiotic stress tolerance are very encouraging. It is essential to analyze in depth the role of PIs in conferring abiotic stress tolerance in transgenic plants. This raises several important questions which need to be addressed to understand the mechanism of PI conferred abiotic stress tolerance, which include: why only a specific set of PIs are induced by a specific kind of stress? Does the overexpression of the PI lead to stress tolerance under which it was induced? Why some PIs are only induced under multiple stress conditions? Are they involved in any crosstalk between the multiple stresses?

The earlier reports suggest that PIs produced in the transgenics inhibit the proteases and thus the stability of vital cellular proteins and enzymes increases leading to enhanced stress tolerance. The PIs are specific to proteases and therefore, which proteases are being inhibited and which proteins and enzymes are escaping the activity of proteases is the key question that needs a thorough analysis.

Studies need to be conducted to know which proteases are being blocked by the overexpressed PI in the transgenic plants and thus, which proteins or enzymes are being protected by the derivative action of the stress induced proteases. It is known that many proteases are induced under stress conditions and PIs specifically inhibit few proteases. Hence, what is the fate of remaining uninhibited proteases? Would they have any deleterious effect on the plant metabolism under stress?

The studies on transgenic PI plants did not report any negative impact of the over expression of the PI in the transgenic plants. Are the PIs in higher concentration not inhibiting any vital proteases that will affect the normal survival of the plants? How can the PIs specifically inhibit the proteases during stress and not during the regular growth? If the PIs are blocking the proteases during normal growth also, then how is the plant negotiating this drawback or does the plant have any alternate mechanisms for controlling the protein turnover or and eliminating utilized proteins. The research in this direction will be exciting and may uncover several important functions of proteases and PIs in plant growth and survival.

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