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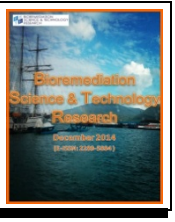

Potential of Plant's Bowman-Birk Protease Inhibitor in Combating Abiotic Stresses: A Mini Review

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ABSTRACT

Bowman-Birk Inhibitor (BBI) is one of the subfamilies of serine protease inhibitors. Numerous studies have shown that in plants, BBI functions as part of their defense mechanism against pathogens and microorganisms. The BBI is also known to have anti-carcinogenic properties. Furthermore, the BBI has been reported to function in controlling abiotic stresses such as salinity and drought stresses.. Abiotic stresses are the major problems in agricultural industry. Therefore, numerous researches have been carried out to characterize the BBI and to determine its roles during biotic and abiotic stresses. This paper presents a review regarding the relationship between Bowman-Birk inhibitor and the plant defensive mechanism against abiotic stresses.

INTRODUCTION

Protease inhibitors (PIs) are molecules that inhibit proteolytic activity of a protease [1] and could be found abundantly in animals, microorganisms, and plant kingdoms [2-9]. In plants, highly concentrated PIs are found in storage tissues, such as tubers and seeds, and in the aerial part of plant [10]. They act as part of plants' defense mechanisms and their productions are peaked during injury or attack by insect or microorganism [11]. PIs would interfere with the digestion process of the insects during infestation. This prevents the consumption and metabolism of essential amino acids in the insects, which eventually lead to their death [12-13]. PIs have been grouped into several families and subfamilies, based on their genomic sequences and the relationship of protein folds of inhibitory domains. The inhibitor domain is a segment in the amino acid sequence that contains a single reactive site after the removal of any part that is not directly involved in the inhibitor activity [14]. Bowman-Birk Inhibitor (BBI) is a protease inhibitor and was first characterized from soybeans by Bowman and Birk [15-16]. In plants, BBI is produced in seeds, legumes, cereals, and wound-induced leaves. Shitan et al. [17] reported that besides their natural role in plant defense, the BBI might also be involved in the abiotic stress response. The BBI protein suppresses the digestion of food in insects or microorganisms due to their inhibitory activities. The inhibitor is induced after a part of plant is bitten and wounded, and its protein expression increases dramatically to protect the plant. A Bowman-Birk inhibitor from *Oryza sativa* has been shown to be involved in resistance against fungal pathogen *Pyricularia oryze* [6]. Cowpea BBI also has been recorded to enhance plants' resistance in transgenic tobacco plants [18]. The growth of

neonate larvae of *Diatraea saccharalis* was significantly retarded when it was fed on transgenic sugarcane's leaf that was overexpressing the BBI [19]. Artificial diet based on soybean BBI showed significantly increased in the mortality rate of nymphal of potato aphid, *Macrosipham euphorbiae* [20]. A study conducted by Prasad et al. [21] had also indicated that BBI from red gram and black gram (*Vigna Mungo*) had insecticidal potential towards lepidopteran insects and significantly reduced the survival rate of *A. janata* larvae. Another research done on *Cajanus cajan*, also showed significantly decrement in the survival rate of both larvae and pupae of *A. janata* [22].

The anticarcinogenic properties in BBI's soybean have long been investigated and it exhibits good suppressing characteristics on carcinogenic processes. It has also been suggested that a large intake of these legumes in human diet that could decreases potential for prostate, colon, breast, and skin cancers is contributed by the presence of the BBI [23]. Furthermore, BBI have indirect involvement in inhibiting trypsin and chymotrypsin enzymes. These proteolytic enzymes have anticarcinogenic properties on many different cell lines [24-25]. The USA Food and Drug Administration (FDA) had granted soybean's BBI to be investigated as a new drug status in 1992. The drug was known as Bowman-Birk Inhibitor Concentration (BBIC) and was in clinical trial for leukoplakia's patients [26]. The soybean's BBI also helps in reducing incidence of optic neuritis, prevents loss of retinal ganglion cell, and significantly improves experimental autoimmune encephalomyelitis (EAE) disease parameters [27].

A study of BBI in *Apios americana* on cancer cell lines revealed that the protease inhibitor of the plant showed an inhibitory effect on proliferation of cells. The chemical modification of arginine or lysine in the inhibitor not only loses the protease-inhibitor activity, but also showed inhibitory effect on cancer cell [28]. The latest study conducted by Fereidunian et al. [29], BBI showed a powerful suppressive effect on tumor progression, both on gastric adenocarcinoma and colorectal adenocarcinoma.

Various medically important BBIs have been isolated and characterized BBI from different species of leguminous and grass plants such as *Apios americana, Cajanus cajan*, *Lathyrus sativus*, *Lupinus albus, Vigna umbellata,* and *Vigna munga* [21,22,28,31-33]. BBI from mung bean has been reported to inhibit dengue viral replication [24,30]. In the study, the researchers had identified the viral protease NS3 as a potential target, which plays a critical role in viral replication. It is worth mentioning that to date there is no effective therapies or vaccines are successfully produced to encounter these problems

Properties and structure of BBI

BBI is classified based on its structural features and inhibitor characteristics. Odani and Ikenaka [34] were the first group to elucidate the BBI based on its covalent structure. **Fig.** 1 shows the structure of BBI. It is a cysteine-rich protein with inhibitory activity against protease and is widely distributed in dicotyledonous and monocotyledonous plant species [35]. BBI from dicotyledonous plants has a single polypeptide chain with molecular mass of 8 kDa [36]. The protein contains two domains and each domain has separate reactive site for their cognate protease. The inhibitor domains work simultaneously, but are independent on each other. The reactive sites in these domains are usually specific to trypsin, chymotrypsin, and elastase [37]. In addition, seven conserved disulfide bonds are present in the active site configurations that also act as stabiliser to these inhibitors [38]. In monocotyledonous plants, two types of BBI have been identified. The first group consists of a single polypeptide chain with molecular mass of 8 kDa, which has only one single reactive site. The second group has two reactive sites, with molecular mass of 16 kDa. Each reactive site is located at the N-terminal of the 8-kDa BBI region, and these two regions have a high intra-molecular identity [39].

Prakash et al. [39] had aligned BBI domains from monocot and dicot plants. Based on the alignment analysis, disulfide bridges occur mostly between two highly conserved cysteine residues, specifically at C_1-C_{14}. The dicot BBI has 14 cysteines while monocot BBI has only 10 cysteines. **Fig.** 2 shows the loss of cysteines residues from monocot plants C_3-C_{13} and C_{10}-C_{11} position when compared to BBI from dicot plants.

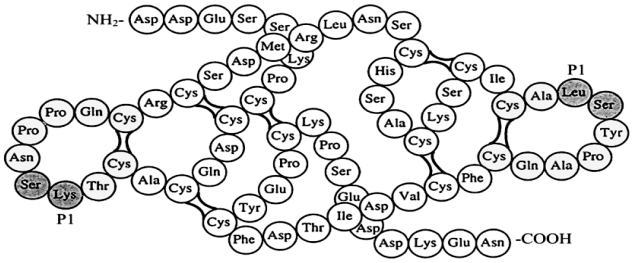

Fig. 1. Structure of Bowman-Birk Inhibitor (lifted from Odani and Ikenaka [40]).

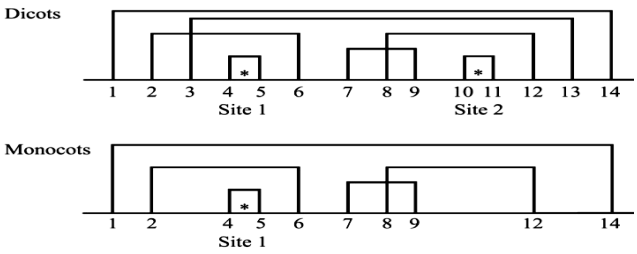

Fig. 2. Disulfide bonds between dicot and monocot plant (lifted from Prakash et al. [39]).

Research on plant abiotic stresses

Stress is defined as mechanical force per unit area applied to an object [41]. In plant, however it is difficult to measure the exact force exerted by stresses [42]. Some biological conditions may be a stress factor for one plant, but it is an optimum condition for another plant. Abiotic stresses can be defined as stresses that caused by environmental factors. The stresses adversely affect survival and reproduction of plants [43]. These factors will suppress the organisms' metabolism and development and can lead to death, in some cases. Table 1 shows the example of abiotic stresses and some researches that have been conducted on abiotic stresses in plants. In agricultural world, environment stresses like cold, drought, and salinity stresses have become the major factors in yield losses. Climatic factors such as extreme temperature,

drought, and salinity are abiotic environment stressors, which are limiting plant growth and development [44]. Wang et al. [45] stated that in the next 25 years, salinisation would result in 30% of non-suitable agricultural land and increased up to 50% in the middle of 21st century. It also has been shown that around 200 million ton of rice is lost due to various natural calamities and 50% of yield lost are caused by drought stress [46].

As these stresses become critical problems in agricultural field, a fast and successful solution to overcome the problems are needed. Numerous methods have been applied for crops improvement. Breeding method is a conventional way to improve crop production and it is a long term and cumbersome process [47]. Throughout the years and advancement in technology, more scientists are opting for agricultural biotechnology compared to conventional breeding for crop improvement (Table 1). Scientists have developed the solution to increase the agricultural production by applying the knowledge based on DNA, which is not possible before [48].

Table 1. List of plant-associated abiotic stresses research.

Abiotic stresses		Main research finding	References
1	Cold	The effect of chilling stresses on the antioxidant defense system in seedling stage of two wheat varieties was investigated. In the experiment, spermidine, one of polyamines compound was used as chill stress. The result showed that injury caused by chilling stress has been reduced significantly with the polyamines introduction.	[49]
		A study has been conducted on cysteinase inhibitor (cystatin) from chestnut in abiotic stresses. From the outcomes, cystatin chestnut (CsC) gene showed it involved in plant defensive to pathogen and pest, as well as to abiotic stresses. The expressions of CsC gene increase as cold and saline shock were applied.	[50]
2	Heat	Heat-stress induced gene was characterized in cabbage. Sequence analysis showed that it exhibited high similarity to the Kunitz type of protease inhibitor. Transcript level increased as the heat stress was applied.	[51]
		Cysteine protease inhibitor from grain amaranth (*Amaranthus hypochondriacus*) was studied during abiotic stresses. The result showed that AhCPI gene responded to salinity, cold, heat and drought stresses. The AhCP1 was suggested to have multiple roles especially as protective agents in combating abiotic stresses.	[52]
3	Salinity	Salt responsive gene (WRSI5) was characterized from salt tolerant wheat. The gene was shown to be conserved BBI domain. The WRSI5 gene expression was highly induced in salt, drought, and oxidative stress.	[53]
		Miraculin- like proteins, Mir1 and Mir2 were studied during salinity stress and insect infestation in citrus plant. The genes showed sequences similar to Kunitz-type protease inhibitor domain. However, the expression patterns depended on the citrus varieties. Mir-1 sequence also showed similar pattern with putative apoplastic cysteine protease (CysP).	[54]
4	Drought	The expression of Bowman-Birk inhibitor in peanut plant under drought stress was studied. AhBBI gene was highly expressed when exposed to water deficit stress.	[55]
		Introgression lines of Huang-Hua-Zhan have been developed and evaluated under drought stress and non-stress. The objective of this study was to develop high yield varieties with high resistances to abiotic stresses.	[56]
5	Flooding	Flooding which has a negative effect in plant growth was studied in two-day-old soybean plant. A flooding-responsive protein from the plant was characterized. Based on the study, the protein was proven to be involved in ethylene signaling.	[57]
		Ubiquitin is one of important role in response to several stresses including flooding stress. The study showed that proteolysis responded to the stress. Ubiquitinated protein showed a decrement after flooding treatment in soybean root and increment after de-submerge. Two of COP9 signalsome (CSN), CSN4, and CSN5 accumulated after the treatment. Hence, the accumulations of the CSN suggested the degradation of ubiquitinated proteins independent of hypoxia caused by flooding.	[58]
6	Radiations	Papain-like cysteine protease (PLCPs) was studied during the application of ozone in maize. PLCPs were shown to be induced by ozone and quicken the senescence process in maize. Based on study, ozone stress was shown to enhance natural senescence processes.	[59]
7	Chemicals and pollutants	A small amount of chemical stress was shown to increase vegetative growth of plants. Glyphosate was used and it significantly increased in crop yield. Application of the glyphosate less than 1% of normal rate would increase barley production around 12-15%.	[60]
		A study on interaction between combination stresses consisting of salinity and other soil chemical stresses in tidal wetland was carried out by this group.	[61]
8	Oxidative stress	Oxidative stress was observed in relation to long-term salinity stress caused by reactive oxygen species (ROS). This study conducted on the susceptible and salt tolerance rice. FL478 showed higher in superoxide dismutase activity. Transcriptomic data showed that higher number of peroxidase genes in FL478 varieties compare to that of salt-susceptible rice.	[62]
		Two putative BBI genes in rice, BIrc1 and BBIrc2 genes were investigated. They showed that the BBIrc1 gene was highly expressed when the oxidative stress was applied.	[63]
10	Nutrient deprivation in soil	This study focused on the effect of silicon in iron deficient soybean and cucumber plants. The outcomes showed that silicon could enhance the growth and alleviate both abiotic and biotic stresses.	[64]
		The effects of nutrients deficiencies on the eco-physiological, biochemical, and growth pattern of pistachio plant were determined. The result was used for guideline in diagnosing nutrient deficiencies of pistachio in commercial nurseries and plantations.	[65]

BBI involvement during salinity stress in wheat

A salt-responsive gene WRSI5 has been shown to be responsible for tolerance to salt stress in wheat (*Triticum aestivum* L.) [53]. This gene contains a Bowman-Birk domain and gene expression profiling of BBI between SR3 and JN177 under salt stress was carried out. WRSI5 gene was characterized from salt tolerant cultivar Shangrong No. 3 (SR3) and salt-susceptible cultivar JN177 [66,67,68].

Full-length coding region (WRSI5) was obtained via 5'/3'smart technology RACE. The sequence contains a coding region for 90-residue cysteine rich polypeptide, with 23-residue signal peptide at N terminus, 10 of the conserved cysteine residues diagnostic for BBIs, and a BBI domain between residues 32 and 87. It also showed ~87% identical to the wheat aluminum stress-related gene *wali5* (AAA50850) and ~86% identical to a protease inhibitor-related protein barley (CAA88619) in amino-acid alignment. **Fig.** 3(A) shows that this gene exhibits a high level of trypsin-inhibiting activity *in vitro*. However, no chymotrypsin inhibitor was observed. This is similarly reported by Ragg et al. [69], which showed BBI suppressed only the trypsin activity. In addition, the WRIS5 gene was more effective as trypsin inhibitor as compared to BBI soybean.

The WRSI5 mRNA was detected in the root of non- stresses SR3 plant using northern blotting and the expression level increased as salt stress was introduced. Transcript level of WRSI5 in different stresses imposed by PEG, H_2O_2, NaCl and $AlCl_3$, also showed similar pattern. As shown in **Fig.** 3(B), strong induction of WRSI5 gene expression was observed in the roots after 1 hour of H_2O_2 stress, 6 hours of salt stress, and 24 hours of PEG or $AlCl_3$ stress.

In plant, peroxidase, H_2O_2 is a defense activator that works together with four proteinase inhibitors. These genes acted as the secondary messengers for the activation of defense genes related [59,70]. The saline, drought, and Al^{3+} ions stresses are related to oxidative stress [60,71], which produced H_2O_2. The H_2O_2 will trigger the expression of several stress-related genes including WRSI5. This signal is also produced during salt, drought or Al^{3+} ions stress.

The growth of SR3 and JN177 rice seedling varieties was investigated under various level of salt stress treatment (0, 150, 200, and 250 mM of NaCl) for 6 days. SR3 seedling was shown to grow better compared to JN177 especially in 150 mM of NaCl [53].

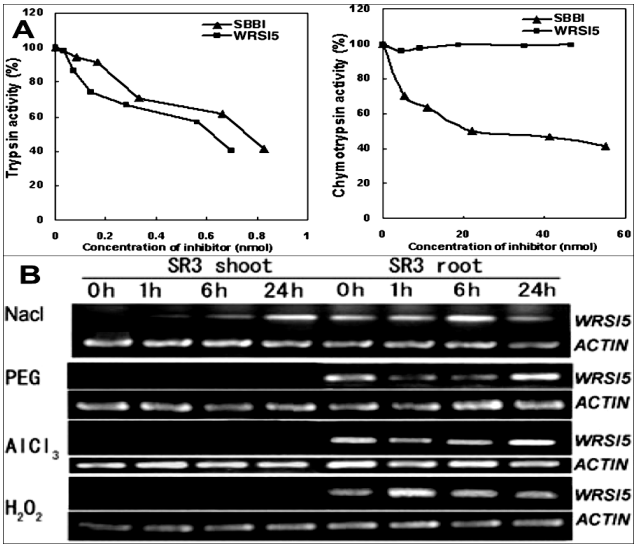

Fig. 3. (A) Trypsin (left) and chymotrypsin (right) in vitro inhibition assay for the WRSI5 fusion protein. (B) WRSI5 expression in root and shoot of SR3 in response to various abiotic stress and different times which are NaCl (200 mm), PEG (15% PEG6000), H_2O_2 (100 mm) and $AlCl_3$ (200 mm) (retrieved from Shan et al. [42,53]).

Fig. 4 shows Na^+ and K^+ content of SR3 and JN177 after 6 days of salt stress. K^+ content of shoots for both SR3 and JN177 plants decreased with the increment of NaCl concentration. In contrast, Na^+ ions content in both types of plants shoots increased as the NaCl concentration increased. Both root and shoot of SR3 and JN177 showed decreasing pattern in K^+ ions content as the level of salinity rose. However, K^+ ions content was found to be less in shoot than in roots. The Na^+ content in both JN177 and SR3 roots increased under the NaCl concentration below 150 mM. The higher salinity stress resulted in lower Na+ content in roots. The K^+/Na^+ ratio of SR3 was higher as compared to that of JN177 by approximately 20%. This is due to the improved level of selectivity for K^+ against Na^+ when ions are transported from root to shoot. The potassium concentration has important roles in defending against biotic and abiotic stresses including salinity stress [72].

BBI involvement during oxidative stress in rice

A novel type of BBI has been identified in the family of rice BBI genes that have three Cys- rich domains at the N-terminus of the sequence. Prior to this, BBI was reported to have only two Cyc-rich domains [6]. Ten BBI genes were identified in rice as a single cluster at chromosome 1. The BBI gene's family showed different expression pattern during seed germination. Lee and Lin [73] reported an inverse relation between the trypsin inhibitor activity and trypsin-like protease activity in senescing rice coleoptiles. However, no studies were conducted on serine activity of the BBI during germination or coleoptiles stages. Yan et al. [63] however studied the gene expression of Bowman-Birk Inhibitor in response to aerobic and hypoxia condition in germination and coleoptiles of rice [74].

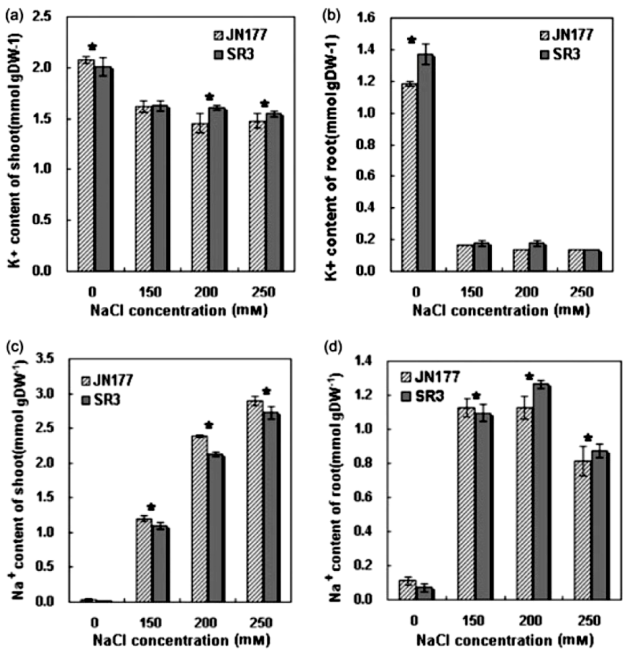

Fig. 4. Na+ and K+ content of SR3 and JN177 after 6 day of salt stress. (a), K+ content of the SR3 and JN177 shoot; (b), K+ content of the SR3 and JN177 root; (c), Na+ content of the SR3 and JN177 shoot; (d), Na+ content of the SR3 and JN177 root. Data are mean _ SE. *Indicates significant differences ($P < 0.05$) (retrieved from Shan et al., 2008) [53].

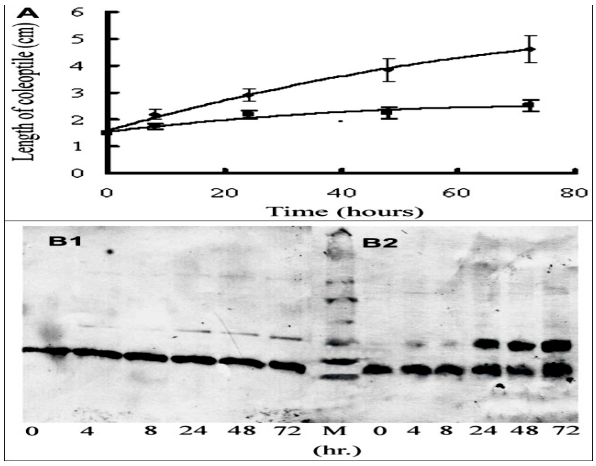

Fig. 5. (A) The measurements of coleoptile growth rate under various conditions and at various time points. 1.5-cm long rice coleoptiles grown in submergence under hypoxia conditions. Then re-grew under aerobic conditions by purging them with atmospheric air (under this condition the oxygen contents was 7.7 mg/L) and anaerobic conditions (under this condition the oxygen content is 1.6 mg/L). (B) Western blotting analysis of rice coleoptiles BBIs. (B1) Coleoptiles grown in anaerobic condition at various time intervals. (B2) The 1.5 cm long coleoptiles grown in hypoxia and in submergence conditions were re-cultivated in anaerobic for various time intervals. (retrieved from Yan et al. [63]).

The result showed that rice coleoptiles that submerged under hypoxia condition elongated much faster as compared to the submergence in aerobic condition. Figure 5(A) shows the coleoptiles growth rate under hypoxia and aerobic condition at different times. The rice coleoptiles in aerobic condition gave a small growth rate as oxidative growth retardation. The oxidative stress changed the metabolic reaction such as degradation of macromolecules. This process led to senescence of coleoptiles as acclimatization process in development stages [75]. Both of this condition exhibited different oxygen availability as observed by Kawai and Uchimiya [76] and formation of BBI was assessed during the coleoptiles development.

Two putative BBI proteins were characterized and named as BBIrc1 (15 kDa) and BBIrc2 (25 kDa). The protein showed the similarity with Rice Bowman-Birk inhibitor 3-3 (RBBI3-3) and Rice Bowman Birk inhibitor (RBBI3-1). Sequence identity was observed for the first 9 amino acid of BBIrc2 with the 37th to 45th amino acid of RBB3-1. Interestingly, similarity was also observed for the 21 amino acid of N-terminal end which was similar with the residue of 119th to 139th of the RBBI3-3 sequence [6]. Using Western blotting analysis, BBIrc1 was shown to be highly expressed in hypoxia condition but remained unchanged under hypoxia condition as shown in **Fig.** 5(B). On the other hand, BBIrc expression started to increase after 8 hour in aerobic condition and reached its peak at 24 hours before remaining constant at 72 hours. However, in hypoxia condition, minimal protein expression was recorded.

BBI is suggested to have potential role in catabolism. During hypoxia condition, plant stops the oxidative catabolism activity and acclimatises to anaerobiosis by switching from oxidative catabolism to fermentative catabolism. Several studies have indicated ethanol as one of the fermentative products and it acts as the oxidative catabolism inhibitor..It is postulated that the induction of protease inhibitor may play important role in regulating protease activities and protein degradation [6]. Yan et al. [63] stated that soluble protein content in coleoptiles grown in hypoxia condition is relatively low than in aerobic condition. Therefore, formation of BBIrc1 may retard proteolytic degradation during hypoxia.

The expression of BBIrc2 has proven that it is induced under aerobic condition. This indicates dependency of the protease inhibitor on the availability of oxygen. Oxygen free radical is generated due to oxygenation of water and the proteins are exposed to proteolytic degradation [77]. As BBI proteins have a large number of disulfide bridges, it may involve in free radical scavenging mechanism. Sulfhydryl-rich proteins such as thioredoxin are widely distributed as small 12-14 kDa oxidoreductases. The proteins play regulatory roles in seed germination and oxidative response [78-79]. It has been proposed that thioredoxins unfold the target proteins by reducing the disulfide bridge making them exposed to proteolytic degradation by cysteine protease.

The induction of BBIrc1 in hypoxia and aerobic condition showed that it might possess another role in the protease activity regulation. Likewise, the BBIrc2 protein is transiently induced when exposure to aerobic condition implying its roles BBI roles in oxidative stress. However, more studies need to be conducted to prove BBI multiple biological functions in rice coleoptiles.

BBI involvement during drought stress in peanut

Arachis hypogaea L. (peanut, groundnut) is an important native oil crop legume from South America which belongs to Leguminosae family and the Papillionacea subfamily [69-71,80,81]. In a research conducted by Drame et al. [55], gene expression of Bowman-Birk inhibitor was studied in response to water deficit, water deficit recovery and phytohormones effect.

Arachis hypogaea L. cultivar Fleur 11 and 73-30, which are tolerant and susceptible varieties to drought, were used in the study of Clavel et al. [82]. Contour-ansel et al. [83] hypothesized that adverse growth condition such as drought will increase various catabolic enzymes including endoprotease. Plant will accumulate specific inhibitor like cystatins to control hydrolytic protein degradations, by these endoprotease [84-85]. In rice for example, a chymotrypsin inhibitor-like protein (OCPI1) is induced during abiotic stresses [75,86]. By assuming that serine endoprotease are regulated by their respective inhibitor during drought, Drame et al. [55] investigated the involvement of BBI in response to drought stress in peanut plant (*Arachis hypogaea L.*).

A novel cDNA (AhBBI) (accession no. DQ011881) encoding a putative BBI was isolated from peanut leaves. The AhBBI expression was studied using real-time-PCR in response to drought and exogenous phytohormones [82]. Full-length coding region was obtained using 5'/3' RACE. The AhBBI has 321 bp-length in site, spinning from an ATG start-codon at position 68 to a TAG stop-codon at position 389. The full-length cDNA of AhBBI consisted of 107 amino acids, as illustrated in **Fig.** 6(A). The AhBBI protein shows a single BBI domain, which has two reactive sites with two arganine residues. The reactive sites are at position 56 and 84 as possible P1 residues and P14 conserved cysteine residues. Analysis of these regions revealed the presence of a 20 amino acid N-terminal of the protein with an N-myristoylation motif at position 1 [55]. The presence of an N-terminal signal sequence with relatively hydrophobic properties in the AhBBI was similar to the other BBI proteins in cowpea, pea, and wheat [87]. Potential casein kinase II phosphorylation sites at positions 41 and 42, and a protein kinase C phosphorylation site at position 93 were also identified.

AhBBI was reported to have high response to Jasmonic acid (JA) with a 40-fold increase in AhBBI expression as compared to control as shown on **Fig.** 6 (B1). However, the AhBBI was repressed by abscisic acid**.** As AhBBI was highly transcripted during the introduction of JA, this proved that AhBBI was involved in defense response against abiotic stress. JA is a signal molecule in plants that involves in initiation of defense mechanism when attacked by pathogens, fungal elicitors, and wounding [88]. High accumulation of AhBBI transcripts was observed when the plants were exposed to water deficit, both in tolerance and susceptible peanut cultivar Fleur11 and 73-30, (**Fig.** 6(B2). Nonetheless, there are some differences in genotype-specific accumulation pattern observed. The tolerant cultivar has higher AhBBI transcript accumulation compared to susceptible cultivar. The cultivar Fleur11 responded more to low water deficit (S1 condition), with 17 times higher but expression decreased gradually until it was similar to control as drought intensified. Limitation of serine protease activity during drought-stress is achieved through down-regulation of a serine protease gene [89]. The over-expression of AhBBI gene in peanut could delay senescence in the tissues by limiting serine protease-induced proteolysis. In conclusion, AhBBI gene does not only play critical role in limiting damage from drought insects, pathogens, and wounds, but it also reduces damage from drought stress through the serine protease activities.

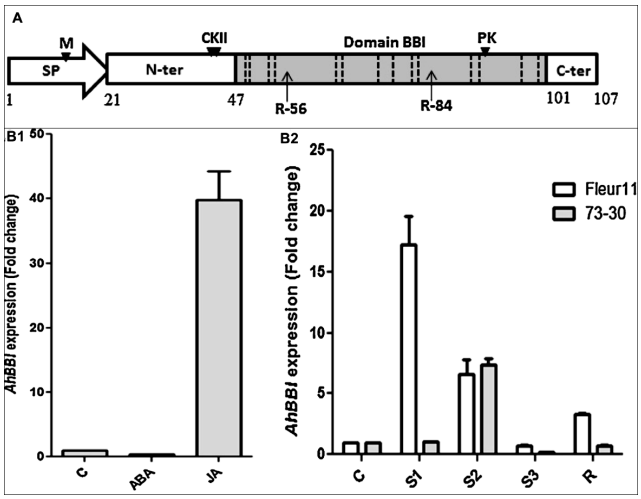

Fig. 6. (A) The full-length cDNA of AhBBI with domain structure of AhBBI, which isolated from peanut leaves. It includes a signal peptide (SP); a BBI domain with 14 conserved cysteine residues (dashed lines), two reactive sites corresponding to arginine residue at position 56 and 84 (R-56 and R-84). N-myristoylation site (M) stated at position 13, casein kinase II phosphorylation sites (CKII) stated at position 41 and 42 and protein kinase C phosphorylation site (PK) at position 93 are also indicated. (B) Expression of AhBBI in peanut leaves treated with hormones or exposed to progressive water deficit. (B1) Expression of AhBBI in separated leaves of peanut cv. Fleur11 treated with water (Control, C), abscissic acid (ABA) or jasmonic acid (JA). (B2) Expression of AhBBI in the leaves of tolerant and susceptible peanut cvs. Fleur 11 and 73–30 under progressive water deficit and rehydration. Water deficit levels correspond to control (C), lightly stressed (S1), moderately stressed (S2), severely stressed (S3) and rehydrated (R). Actin was used to standardize each reaction run and expression is presented as relative fold change compared to control (Retrieved from Drame et al. [55]).

CONCLUSION

BBI shows a great response towards tolerance to abiotic stresses especially a major stress like salinity and drought stress for plant production. These researches will open new possibilities in introducing recombinant protease inhibitors in plants, which could defend the plants from abiotic stresses.

REFERENCES

[1] Habib H, Fazili KM. Protease inhibitor: A defense strategy in plant. Biotechnol Mol Biol. 2007; 2(3):068–085.

[2] Valueva TA, Mosolov VV. Role of inhibitors of proteolytic enzymes in plant defense against phytopathogenic microorganisms. Biochem. 2004; 69(11):1305–1309.

[3] Christeller JT. Evolutionary mechanisms acting on proteinase inhibitor variability. FEBS J. 2005; 272:5710–5722.

[4] Haq SK, Atif SM, Khan RH. Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: Natural and engineered phytoprotection. Arch Biochem Biophys. 2004; 43(1):145–159.

[5] Rahbe Y, Ferrasson EH, Rabesona L. Toxicity to the pea aphid *Acyrthosiphon pisum* of anti-chymotrypsin of anti-chymotrypsin isoforms and fragments of Bowman-Birk protease inhibitors from pea seeds. Insect Biochem Mol Biol. 2003; 33:299–306.

[6] Qu LJ, Chen J, Liu M, Pan N, Okamoto H, Lin Z, Li C, Li D, Wang J, Zhu G, Zhao X, Chen X, Gu H, Chen Z. Molecular cloning and functional analysis of a novel type of Bowman-Birk inhibitor gene family in rice. Plant Physiol. 2003; 133:560–570.

[7] Deshimaru M, Yoshimi S, Shioi S, Terada S. Multigene family for Bowman-Birk type proteinase inhibitors of wild soya and soybean: The presence of two BBI-A genes and pseudo genes. J-Stage. 2004; 68:1279–1286.

[8] Otlewski J, Jelen F, Zakrzewska M, Oleksy A. The many faces of protease protein inhibitor interaction. The EMBO J. 2005; 24:1303–1310.

[9] Ivanov D, Emonet C, Foata F, Affolter M, Delly M, Fisseha M, Blum-Sperisen S, Kochhar S, Arigoni F. A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase like serine proteases. J Biol Chem. 2006; 281(25):17246–17252.

[10] De Leo F, Volpicella M, Licciulli F, Liuni S, Gallerani R, Ceci LR. PLANT-PIs: A database for plant protease inhibitors and their genes. Nucleic Acids Res. 2002; 30(1):347–348.

[11] Ryan CA. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. Annu Rev Phytopathol. 1990; 28:425–449.

[12] Clemente A, Domoney C. Biological significance of polymorphism in legume protease inhibitors from the Bowman-Birk family. Curr. Protein Pept. Sci. 2006; 7:201–216.

[13] Lawrence PK, Koundal KR. Plant protease inhibitors in control of phytophagous insects. Electron J Biotechnol. 2002; 5(1):93–109.

[14] Rawlings ND, Tolle DP, Barrett AJ. Evolutionary families of peptidase inhibitors, J Biochem. 2004; 378:705–716.

[15] Bowman DE. Differentiation of soybean antitryptic factor. Proc Soc Exp Biol Med. 1946; 63: 547–550.

[16] Birk Y, Gertler A, Khalef S. A pure trypsin inhibitor from soya bean. Biochem J. 1963; 87:281–284.

[17] Shitan N, Horiuchi K, Sato F, Yazaki K. Bowman-birk proteinase inhibitor confers heavy metal and multiple drug tolerance in yeast. Plant Cell Physiol. 2007; 48:193–197.

[18] Hilder VA, Barker RF, Samour RA, Gatehouse AM, Gatehouse JA, Boulter D. Protein and cDNA sequences of Bowman-Birk protease inhibitors from the cowpea (*Vigna unguiculata* Walp). Plant Mol Biol 1989; 13:701–710.

[19] Falco MC, Silva-Filho MC. Expression of soybean proteinase inhibitors in transgenic sugarcane plants: Effects on natural defense against *Diatraea saccharalis.* Plant Physiol Biochem. 2003; 41:761–766.

[20] Azzouza H, Cherquia A, Campana EDM, Rahbe´b Y, Duportb G, Jouaninc L, Kaiserd L, Giordanengoa P. Effects of plant protease inhibitors, oryzacystatin I and soybean Bowman–Birk inhibitor, on the aphid *Macrosiphum euphorbiae* (Homoptera, Aphididae) and its parasitoid *Aphelinus abdominalis* (Hymenoptera, Aphelinidae). J Insect Physiol. 2005; 51:75–86.

[21] Prasad ER, Dutta-Gupta A, Padmasree K. Purification and characterization of a Bowman-Birk proteinase inhibitor from the seeds of black gram (*Vigna mungo*). Phytochem. 2010; 71:363–372.

[22] Swathi M, Lokya V, Swaroop V, Mallikarjuna N, Kannan M, Dutta-Gupta A, Padmasre K. Structural and functional characterization of proteinase inhibitors from seeds of *Cajanus cajan* (cv. ICP 7118) Plant Physiol Biochem. 2014; 83:77–87.

[23] Kennedy AR. The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. Am J Clin Nutr. 1998; 68:1406s–1412s.

[24] Billings PC, Habres JM. A growth-regulated protease activity that is inhibited by the anticarcinogenic Bowman-Birk protease inhibitor. Proc Natl Acad Sci USA 1992; 89:3120–3124.

[25] Kennedy AR, Wan XS. Effects of the Bowman-Birk inhibitor on growth, invasion, and clonogenic survival of human prostate epithelial cells and prostate cancer cells. Prostate. 2002; 1:50(2):125–33.

[26] Armstrong WB, Kennedy AR, Wan XS, Tayor TH, Nguyen QA, Jensen J, Thompson W. Clinical modulation of oral leukoplakia and protease activity by Bowman-Birk inhibitor concentrate in a phase IIa chemoprevention trial. Clin Cancer Res 2000; 6:4684–4691.

[27] Touil T, Ciric B, Ventura E, Shindler KS, Gran B, Rostami A. Bowman–Birk inhibitor suppresses autoimmune inflammation and neuronal loss in a mouse model of multiple sclerosis, J Neurol Sci. 2008; 217:191–202.

[28] Zhang Y, Zhou C, Tang C, Yu, Kouzuma Y, Yonekura Y. Effect of AATI, a Bowman-Birk type inhibitor from *Apios americana*, on proliferation of cancer cell lines. Food Chem. 2011; 218:909–915.

[29] Fereidunian A, Sadeghalvad M, Oscoie MO, Mostafaie A. Soybean Bowman-Birk protease inhibitor (BBI): Identification of the mechanisms of BBI suppressive effect on growth of two adenocarcinoma cell lines: AGS and HT29. Arch Med Res. 2014; 45:455–461.

[30] Murthy HM, Judge K, De Lucas L, Padmanabhan R. Crystal structure of Dengue virus NS3 protease in complex with a Bowman-Birk inhibitor: Implications for flaviviral polyprotein processing and drug design. J Mol Biol. 2000; 301:759–767.

[31] Katoch R, Singh SK, Thakur N, Dutt S, Yadav SK, Shukle R. Cloning, characterization, expression analysis and inhibition studies of a novel gene encoding Bowman–Birk type protease inhibitor from rice bean, Gene. 2014; 546:342–351.

[32] Scarafoni A, Consonni A, Galbusera V, Negri A, Tedeschi G, Rasmussen P, Magni C, Duranti M. Identification and characterization of a Bowman–Birk inhibitor active towards trypsin but not chymotrypsin in *Lupinus albus* seeds. Phytochem. 2008; 69:1820–1825.

[33] Paola DD, Blanco E, Pierri CL, Sonnante G. Isolation and characterization of novel variants of BBI coding genes from the legume *Lathyrus sativus*. Plant Physiol Biochem. 2012; 57:45–53.

[34] Odani S, Koide T, Ono T. Wheat gram trypsin inhibitor. Isolation and structural characterization of single-headed and double-headed inhibitors of the Bowman-Birk type. J Biochem. 1986; 100(4):975–983.

[35] Lin YH, Li HT, Huang YC, Hsieh YC, Guan HH, Liu MY, Chang T, Wang AHJ, Chen CJ. Purification, crystallization and preliminary Xray crystallographic analysis of rice Bowman-Birk inhibitor from *Oryza sativa*. Acta Cryst F. 2006; 62:522–524.

[36] Birk Y. The Bowman-Birk inhibitor. Trypsin and chymotrypsin inhibitor from soybeans. Int J Pept Protein Res. 1985; 25:113–131.

[37] Qi RF, Song Z, Chi C. Structural features and molecular evolution of Bowman-Birk protease inhibitors and their potential application: BBA. 2005; 37(5):283–292.

[38] Lin GD, Bode W, Huber R, Chi CW, Engh RA. The 0.25 nm X-ray structure of the Bowman-Birk type inhibitor from mung bean in ternary complex with porcine trypsin. Eur J Biochem. 1993; 212:549–555.

[39] Prakash B, Selvaraj S, Murthy MRN, Sreerama YN, Rao DR, Gowda LR. Analysis of the amino acid sequences of plant Bowman-Birk inhibitors. J Mol Evol. 1996; 42:560–569.

[40] Odani S, Ikenaka T. Scission of soybean Bowman-Birk proteinase inhibitor into two small fragments having either trypsin or chymotrypsin inhibitor activity. J. Biochem (Tokyo). 1973; 74:857–860.

[41] Heribert H (ed.). Plant stress biology: From genomics to systems biology. 2009. Paris: Wiley-Blackwell

[42] Jones HG, Flowers TJ, Jones MB. Plants under Stress. Cambridge Univ. Press, Cambridge. 1989.

[43] Chinnusamy, V., Jan-Kang Zhu. Epigenetic regulation: Chromatin modeling and small RNAs. Abiotic Stress Adaptation in Plants. 2010: 217-241

[44] Krasensky J, Jonak C. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks, J Exp Bot. 2012; 63:1593–1608.

[45] Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 2003; 218:1–14.

[46] Chen TH, Murata N. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol. 2002; 5(3):250–257.
[47] Abdullah SNA, Panjaitan SB, Aziz MA, Sariah M, Omar O. Somatic embryogenesis from scutellar embryo of *Oryza sativa* L. var. MR219. Pertanika J Trop Agric Sci. 2009; 32(2):185–194.
[48] J. Piesse, J., Thirtle, C. Agricultural R&D, technology and productivity. Philos Trans R Soc Lond B Biol Sci. 2010. 365(1554): 3035–3047
[49] Abdel Kader DZ, Saleh AAH, Elmeleigy SA, Dosoky NS. Chilling-induced oxidative stress and polyamines regulatory role in two wheat varieties. JTUSCI. 2011; 5:14–24.
[50] Prenas M, Sa'nchez-Monge R, Salcedo G. Biotic and abiotic stress can induce cystatin expression in chestnut. FEBS Lett. 2000; 467:206–210.
[51] Annamalai P, Yanagihara S. Identification and characterization of a heat-stress induced gene in cabbage encodes a kunitz type protease inhibitor. J Plant Physiol. 1999; 155(2):226–233.
[52] Valdés-Rodríguez S, Guerrero-Rangel A, Melgoza-Villagómez C, Chagolla-López A, Delgado-Vargas F, Martínez-Gallardo N, Sánchez-Hernández C, Délano-Frier J. Cloning of a cDNA encoding a cystatin from grain amaranth (*Amaranthus hypochondriacus*) showing a tissue-specific expression that is modified by germination and abiotic stress. Plant Physiol Biochem. 2007; 45:790–798.
[53] Shan L, Li C, Chen F, Zhao S, Xia G. A Bowman-Birk type protease inhibitor is involved in the tolerance to salt stress in wheat. Plant Cell Environ. 2008; 31:1128–1137.
[54] Podda A, Simili M, Del Carratore R, Mouhaya W, Morillon R, Maserti BE. Expression profiling of two stress-inducible genes encoding for miraculin-like proteins in citrus plants under insect infestation or salinity stress. J Plant Physiol. 2014; 171:45–54.
[55] Dramé KN, Passaquet C, Repellin A, Fodil YZ. Cloning, characterization and differential expression of a Bowman–Birk inhibitor during progressive water deficit and subsequent recovery in peanut (*Arachis hypogaea*) leaves. J Plant Physiol. 2013; 170:225–229.
[56] Wang Y, Zhang L, Nafisah A, Zhu L, Xu J, Li Z. Selection efficiencies for improving drought/salt tolerances and yield using introgression breeding in rice (*Oryza sativa* L). The Crop J. 2013; 1:134 –142.
[57] Yin X, Sakata K, Nanjo Y, Komatsu S. Analysis of initial changes in the proteins of soybean root tip under flooding stress using gel-free and gel-based proteomic techniques. J Proteomics. 2014; 106:1–16.
[58] Yanagawa Y, Komatsu S. Ubiquitin proteasome-mediated proteolysis is involved in the response to flooding stress in soybean roots, independent of oxygen limitation. Plant Sci. 2012; 185:250–258.
[59] Ahmad R, Zuily-Fodil Y, Passaquet C, Bethenod O, Roche R, Repellin A. Identification and characterization of MOR-CP, a cysteine protease induced by ozone and developmental senescence in maize (*Zea mays* L.) leaves. Chemosphere. 2014; 108:245–250.
[60] Cedergreen N, Felby C, Porter JR, Streibig JC. Chemical stress can increase crop yield. Field Crops Res. 2009; 114:54–57.
[61] Quijano-Guerta C, Kirk GJD. Tolerance of rice germplasm to salinity and other soil chemical stress in tidal wetlands. Field Crops Res. 2002; 76:111–121.
[62] Senadheera P, Tirimanne S, Maathuis FJM. Long term salinity stress reveals variety specific differences in root oxidative stress response. Rice Sci, 2012; 19:36–43.
[63] Yan KM, Chang T, Soon SA, Huang YF. Purification and characterization of Bowman-Birk protease inhibitor from rice coleoptiles. J Chin Chem Soc. 2009; 56:949–960.
[64] Gonzalo MJ, Lucena JJ, Hernández-Apaolaza L. Effect of silicon addition on soybean (*Glycine max*) and cucumber (*Cucumis sativus*) plants grown under iron deficiency. Plant Physiol Biochem. 2013; 70:455–461.
[65] Afrousheh M, Ardalan M, Hokmabadi H. Nutrient deficiency disorders in *Pistacia vera* seedling rootstock in relation to eco-physiological, biochemical characteristics and uptake pattern of nutrients. Sci Hort. 2010; 124:141–148.
[66] Chen S, Quan T, Xia G, Jin Y, Chen H, Xiang F. Introgression of salt-tolerance from somatic hybrids between common wheat and *Thinopyrum ponticum*. Plant Sci. 2004; 167:773–779.
[67] Xia G, Xiang F, Zhou A, Wang H, Chen H. Asymmetric somatic hybridization between wheat (*Triticum aestivum* L.) and *Agropyron elongatum* (Host). Nevishi Theor Appl Genetics. 2003; 107:299–305.
[68] Shan L, Zhao S, Chen F, Xia G. Screening and localization of SSR markers related to salt tolerance of somatic hybrid wheat Shanrong No. 3. Sci Agric Sinica. 2006; 39:225–230.
[69] Ragg EM, Galbusera V, Scarafoni A, Negri A, Tedeschi G, Consonni A, Sessa F, Duranti M. Inhibitory properties and solution structure of a potent Bowman-Birk protease inhibitor from lentil (*Lens culinaris* L) seeds. FEBS J. 2006; 273:4024– 4039.
[70] Orozco-Cárdenas ML, Narváez-Vásquez J, Ryan CA. Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell. 2001; 13:179–191.
[71] Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2002; 7:405–410.
[72] Wang M, Zheng Q, Shen Q, Guo S. The critical role of potassium in plant stress response. Int J Mol Sci. 2013; 14:7370–7390.
[73] Lee TM, Lin YH. Trypsin inhibitor and trypsin-like protease activity in air- or submergence-grown rice (*Oryza sativa* L.) coleoptiles. Plant Sci. 1995; 106:43–54.
[74] Chang T, Kuo MD, Khoo KH, Inoue S, Inoue Y. Developmentally regulated expression of a peptide: N-glycanase during germination of rice seeds (*Oryza sativa*) and its purification and characterization. J Biol Chem. 2000; 275:129–134.
[75] Inada N, Sakai A, Kuroiwa H, Kuroiwa T. Three-dimensional of the senescence program in rice (*Oryza Sativa* L.) coleoptiles. Investigation of tissues and cells by fluorecence microscopy. Planta. 1998; 205:153–164.
[76] Kawai M, Uchimiya H. Coleoptile senescence in rice (*Oryza sativa* L.). Ann Bot. 2000; 86:405–414.
[77] Das A, Uchimiya H. Oxygen stress and adaptation of a semi-aquatic plant: Rice (*Oryza sativa*). J Plant Res. 2002; 115:315–320.
[78] Yano H, Kuroda, M. Disulfide proteome yields a detailed understanding of redox regulation: a model study of thioredoxin-linked reaction in seed germination. Proteomics. 2006; 6:294–300.
[79] Zeller T, Klung G. Thioredoxins in bacteria: Functions in oxidative stress response and regulation of thioredoxin genes. Naturwissenschaften. 2006; 93:259–266.
[80] Chen N, Yan Q, Hu D, Pan L, Chi X, Chen M, Yang Z, Wang T, Wang M, He Y, Yu S. Gene expression profiling and identification of resistance genes to low temperature in leaves of peanut (*Arachis hypogaea* L.) Sci Hort. 2013; 169:214–225.
[81] Sharma KK, Mathur, PB. Peanut (*Arachis Hypogaea* L.). Methods Mol Biol. 2006; 58:343–347.
[82] Clavel D, Dramé KN, Roy-Macauley H, Braconnier S, Laffray D. Analysis of early responses to drought associated with field drought adaptation in four Sahelian groundnut (*Arachis hypogaea* L.) cultivars. Environ Exp Bot. 2005; 54:219–30.
[83] Contour-Ansel D, Torres-Franklin ML, Zuily-Fodil Y, Cruz de Carvalho MH. An aspartic acid protease from common bean is expressed 'on call' during water stress and early recovery. J Plant Physiol. 2010; 167:1606–12.
[84] Diop NN, Kidric M, Repellin A, Gareil M, Arcy Lameta, AD Pham-Thi AT. A multicystatin is induced by drought-stress in cowpea (*Vigna unguiculata* (L.) Walp.) in leaves. FEBS Lett. 2004; 577:545–50.
[85] Megdiche W, Passaquet C, Zourrig W, Zuily-Fodil Y, Abdelly C. Molecular cloning and characterization of novel cystatin gene in leaves Cakile maritima halophyte. J Plant Physiol. 2009; 166:739–49.

[86] Huang Y, Xiao B, Xiong L. Characterization of a stress responsive proteinase inhibitor gene with positive effect in improving drought resistance in rice. Planta. 2007; 226:73–85.
[87] Domoney C, Welham T, Sidebottom C, Firmin JL. Multiple isoforms of Pisum trypsin inhibitors result from modification of two primary gene products. FEBS Lett. 1995; 360:15–20.
[88] Li L, Li C, Lee GI, Howe GI. Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc Natl Acad Sci. 2002; 99:6416–21.
[89] Dramé KN, Clavel D, Repellin A, Passaquet C, Zuily-Fodil Y. Water deficit induces variation in expression of stress-responsive genes in two peanut (*Arachis hypogaea* L.) cultivars with different tolerance to drought. Plant Physiol Biochem. 2007; 45:236–243.
[90] Etienne P, Desclos M, Gou LL, Gombert J, Bonnefoy J, Maurel K. N-protein mobilisation associated with leaf senescence process in oilseed rape is concomitant with the disappearance of trypsin inhibitor activity. Funct Plant Biol. 2007; 34:895–906.