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Aspartic protease in leaves of common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* L. Walp): enzymatic activity, gene expression and relation to drought susceptibility

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Abstract Four cultivars of related species, common bean and cowpea, which exhibit different degrees of drought resistance, were submitted to water stress by withholding irrigation. Drought induced an increase in endoproteolytic activity, being higher in susceptible cultivars (bean) than in tolerant ones (cowpea). An aspartic protease activity was found to be strongly induced especially in bean. From a cowpea leaf cDNA library, a full length aspartic protease precursor cDNA was obtained. Transcript accumulation in response to water stress indicated that the expression of the gene was constitutive in cowpea and transcriptionally up-regulated in bean. The results showed that drought-tolerant and drought-susceptible bean plants differ regarding aspartic protease precursor gene expression.

Key words: Drought stress; Endoproteolytic activity; Aspartic protease precursor cDNA; Cowpea (*Vigna unguiculata* L. Walp); Common bean (*Phaseolus vulgaris* L.)

1. Introduction

Aspartic proteases (APs, EC 3.4.23) are members of a class of endopeptidases with acidic pH optima that are inhibited by pepstatin A. APs include animal pepsin, renin and cathepsin D, fungal penicillopepsin, yeast protease A, and HIV protease [1]. They have a conserved three dimensional structure with a substrate binding cleft between the two lobes of the structure. Two conserved Asp residues are specifically involved in the catalytic cleavage of peptide bonds between amino acid residues with large hydrophobic side chains [1,2]. APs are synthesized as zymogens and they are self-processed to yield the active enzyme [1].

Little is known about the biological function of APs in plants [3,4]. It has been suggested that an AP could be involved in the digestion of insects in *Nepenthes* [5], in the degradation of plant proteins in response to pathogens [6,7], during development processes [8–10] and senescence [11,12]. In mammalian cells, the lysosomal pathway is responsible for the enhanced protein degradation observed under stress conditions [13].

Protease activities involved in plant response to water stress have received little attention particularly in legumes. Taking this into account, we have used a previously developed plant system [14,15] to check whether or not endoproteases (protease) are involved in water stress plant response and if so to determine if it is related to plant drought susceptibility.

The plant system consists of related bean plants (common bean and cowpea) which show different drought resistance capacities under field conditions [14]. Previous physiological studies have shown that the cowpea cultivars establish adaptive strategies under drought which are absent in the more susceptible bean species [16,17]. Drought resistance (or susceptibility) of these plants correlated well with their tolerance at the cell level, in terms of membrane integrity [14] and membrane lipid degradation [18,19]. Water deficit results in a loss of proteins in soluble, membrane and chloroplast cell fractions, being dependent on the intensity of water stress and being higher in susceptible bean cultivars than in tolerant cowpea cultivars [15].

Proteolysis during plant senescence is well documented [20,21], but little is known about the nature of endoproteases involved in water stress response [15,22], and their physiological significance. In order to characterize the endoproteases, we have assayed specific endoproteolytic activities in soluble leaf extracts using class-specific inhibitors. The results showed that an AP is involved in the degradation process induced by water stress. To identify the gene, a cDNA library from cowpea leaves [19] was screened. A cDNA encoding a putative full length AP precursor was isolated. The accumulation of the cowpea aspartic precursor mRNA was studied in the case of the four cultivars of the plant system, submitted to various degrees of water deficit.

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Abbreviations: ABA, abscisic acid; AP, aspartic protease; pCMB, *para*-chloromercuribenzoate; PMSF, phenylmethane sulfonyl fluoride; TCA, trichloroacetic acid; *Vu*, *Vigna unguiculata*

2. Materials and methods

2.1. Plant culture and treatments

The plant system consists of related diploid autogamous bean plants of the same family (*Fabaceae*) and tribe (*Phaseolidae*), which

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show different drought resistance capacities under field conditions, namely two cultivars of cowpea – *Vigna unguiculata* L. Walp cv. EPACE-1 and cv. IT83D and two cultivars of common bean – *Phaseolus vulgaris* L. cv. Carioca and cv. IPA. Cowpea cultivars are more tolerant than bean cultivars: EPACE-1 > IT83D > IPA > Carioca [15,16].

Plants were grown in green house as previously described [15]. Drought stress was induced by withholding watering for 7–10 days in 21 day old plants. Experiments were carried out on the second fully expanded leaf. Three stress levels of water deficit were defined: S_1, S_2, S_3: $\psi_w = -1.0$, -1.5, -2.0 MPa, respectively (control plants C: $\psi_w = -0.3$ MPa). They were measured using a pressure chamber (PMS ECS Instruments) [23]. In recovery experiments, S_2 plants were re-hydrated (R) and harvested 24 h later ($\psi_w = -0.3$ MPa). Abscissic acid (ABA) treatment was carried out on detached leaves with petiole soaked in 0.1 mM ABA, 10 mM Tris buffer pH 7.0 for 24 h, control in buffer [24].

2.2. Endoproteolytic activity

Leaves (1 g) were frozen in liquid nitrogen with insoluble polyvinylpyrrolidone (0.2 g g^{-1} FW), homogenized in 5 ml 50 mM Tris–HCl buffer pH 7.5, 5% (v/v) 2-morpholinoethane-sulfonic acid and filtered (nylon nets 60 and 100 μm pore sizes, Monyl, Polylabo, Strasbourg, France). The filtrate was centrifuged (15 000 × *g*, 10 min, 4°C, Beckman Ultracentrifuge L5.50R) and the supernatant was used as crude enzyme extract (CE). Endoproteolytic activities were assayed using ^{14}C-methylated casein [25] as a substrate (10.6 μCi (mg protein)$^{-1}$, Sigma) at pH 4.5. 20 μl of CE and 80 μl ^{14}C-methylated casein in acetate–acetic acid buffer pH 4.5 were mixed and incubated during 3 h at 37°C. The reaction was stopped by adding 10 μl of 2% bovine serum albumin (BSA) (w/v) and 90 μl of 10% trichloroacetic acid (TCA) (w/v). The TCA-soluble radioactivity was measured in the supernatant (Liquid scintillation analyzer 1600 CA, Packard). The endoproteolytic activity was expressed as μg of ^{14}C-methylated casein hydrolyzed (mg protein)$^{-1}$ h^{-1}.

2.3. Inhibition of endoprotease activity

Experiments were carried out using control and S_2 plants of bean cv. Carioca. Class-specific protease inhibitors [26] were used: 2 mM phenylmethane sulfonyl fluoride (PMSF, serine protease inhibitor), 0.15 mM pepstatin A (specific AP inhibitor), 1 mM *para*-chloromercuribenzoic acid (pCMB, cysteine protease inhibitor) and 10 mM EDTA (metalloprotease inhibitor). CE was partially purified (PE) by precipitation with 80% (w/v) ammonium sulfate, desalted by Sephadex G-25 (Pharmacia, Uppsala, Sweden) gel filtration followed by anion-exchange chromatography (Mono Q HR 5/5, Pharmacia, Uppsala, Sweden). PEs were preincubated separately with inhibitors for 1 h and 30 min at 4°C at the appropriate pH optima and endoproteolytic activities were measured using ^{14}C-methylated casein as above. Results are expressed as percentage inhibition defined as the difference between the enzymatic activity after incubation without inhibitor (EA_0) and that with inhibitor (EA_i), expressed in percent $[(EA_0 - EA_i)/EA_0] \times 100$.

2.4. AP activity assay

AP activities were determined in leaf enzyme extracts (LE) which were brought to a final volume of 1 ml with 0.1 M sodium acetate–HCl pH 3.0 buffer. The reaction was initiated by adding a synthetic substrate Pro-Thr-Glu-Phe-(NO_2-Phe)-Arg-Leu (Novabiochem, Switzerland) to a final concentration of 0.3 mM, according to [27]. After 15 min at 37°C, the absorbance (*A*) was measured at 310 nm (Perkin Elmer). AP activity was expressed in ΔA_{310} (mg protein)$^{-1}$ min^{-1}. Protein content was determined using the Bio-Rad protein assay reagent (Bio-Rad, Richmond, USA [28]) with BSA as a standard.

2.5. cDNA cloning and sequence analysis

Primers corresponding to AP precursor consensus regions were used in PCR amplifications using cDNA of *Vu* S_2 as a template (cDNA synthesis kit, Amersham). The amplified DNA fragment was purified (Wizard PCR Prep, Promega) and cloned in the pCRII plasmid (T/A cloning kit, Invitrogen). A λ Zip-Lox (Gibco-BRL) cDNA library constructed from mRNA of cowpea plants [19] was screened and the longest positive plasmid clone was obtained after excision in vivo in DH10B(ZIP) *Escherichia coli* cells. Sequencing was carried out on one strand using the dideoxy chain-termination method [29] with the Oncor sequencing kit (Appligene). The other strand was sequenced by ESGS (Paris, France). Results were analyzed with the PC/gene program (Intelligenetics Inc., Mountain View, CA, USA).

2.6. mRNA isolation and Northern blotting

Bean and cowpea leaves (6 g FW) frozen in liquid nitrogen were used for total RNA extraction [30]. mRNA was obtained using Oligotex columns (Qiagen) according to manufacturer's instructions. 3 μg mRNA of each treatment was separated on a 1% agarose-formaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham) and hybridized with a ^{32}P-labeled DNA probe of 330 bp obtained by PCR cDNA amplification between oligonucleotides corresponding to amino acids 20–130 of cowpea AP cDNA *VuAP1*. RNA loading was checked by re-probing the membranes with *nt*S19 (encoding S19 ribosome protein from *Nicotiana tabacum*).

3. Results

3.1. Leaf endoprotease activity under water stress

In control plants (water potential: $\psi_w = -0.3$ MPa), activities of endoproteases assayed using ^{14}C-methylated casein were not significantly different in bean and cowpea cultivars (Fig. 1). In mildly stressed plants (S_2: $\psi_w = -1.5$ MPa), withholding irrigation led to increased activity in bean cv. Carioca (+235%), IPA (+119%) and cowpea cv. IT83D (+95%). Lower values were obtained for the more tolerant cv. EPACE-1 (+58%).

3.2. Characterization of an AP activity

The percentage of inhibition of proteolytic activities, induced by class-specific inhibitors, was determined in watered and stressed plants in the case of the more drought-susceptible cultivar of the plant system, cv. Carioca. Proteases are classified as cysteine (CP), aspartic (AP), serine (SP) and metalloproteases (MP) as defined by Barrett [26]. The experiments were conducted using partially purified (PE) leaf extracts of control ($\psi_w = -0.3$ MPa) and S_2 ($\psi_w = -1.5$ MPa) plants of bean Carioca (Fig. 2). EDTA (metalloprotease inhibitor) did not result in any change in percentage inhibition of endoproteolytic activity, in either watered or non-watered plants, thus excluding significant contributions from metalloproteases in

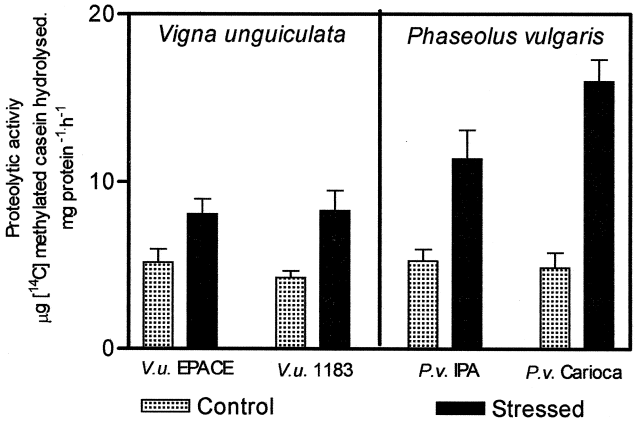

Fig. 1. Endoproteolytic activities in crude leaf extracts from control and water-stressed *Phaseolus* and *Vigna* plants. The activities were measured at pH 4.5 and expressed as μg ^{14}C-methylated casein hydrolyzed (mg protein)$^{-1}$ h^{-1}. The leaf extracts were obtained from control (C: $\psi_w = -0.3$ MPa) and water-stressed (S_2: $\psi_w = -1.5$ MPa) plants. Values are means of three replicates from a representative experiment.

leaf extracts. Serine and cysteine protease activities, as identified by their respective inhibitors, PMSF and pCMB, decreased in stressed plants as compared to controls. Pepstatin A, a class-specific AP inhibitor, resulted in a weak inhibition of endoproteolytic activity in leaf extracts of watered plants, indicating a low level of AP activity in unstressed plants. However, in the stressed plants, 25% of the total proteolytic activity was inhibited by pepstatin A, showing that the level of AP activity is dramatically increased in bean Carioca leaves under these conditions of water deficit.

3.3. AP activity under water stress

Experiments were performed on control ($\psi_w = -0.3$ MPa) and S_2 ($\psi_w = -1.5$ MPa) bean and cowpea plants. A peptide substrate specific for APs, Pro-Thr-Glu-Phe-(NO_2-Phe)-Arg-Leu, was used to assay their activity in partially purified leaf extracts (without proteins which precipitate at pH 3.0) (Fig. 3). The results showed that water deficit significantly stimulated AP activity, the stimulation being higher with increasing plant sensitivity to drought stress (see Section 2).

3.4. Cloning and sequence analysis of a cDNA encoding a putative AP precursor of cowpea

Primers, 5′-GGNTGYGCTGCTATHGCTGA (sense) and 5′-CCCATRAANACRTCNCC (antisense), where N = A/C/G/T, H = A/T/C, R = A/G, Y = C/T, were designed according to AP precursor consensus sequences of barley (GenBank accession no. X56136), cardoon (X81984) and rice (D12777). The 612 bp long probe was used to screen a cDNA library constructed from cowpea leaf [19]. A full length clone of 1842 bp was isolated, sequenced and referenced as *VuAP1* (GenBank accession number U61396). The open reading frame encodes a 513 amino acid protein with a calculated molecular mass of 55.4 kDa and a predicted p*I* of 5.6. *VuAP1* has highly conserved regions in the two catalytic domains with identical position and length as compared to the conserved regions of barley (X56136) [31], cardoon (X81984) [32], *Brassica napus* (U55032) [33], *Arabidopsis thaliana* (U51036) [33] and rice (D12777) [9]. *VuAP1* showed 74–78% sequence identity with these plant AP precursors. A signal was detected at the amino-terminus of *VuAP1*, known to be responsible for the targeting of the protein into the cell vacuole. The prediction of the cleavage site (Psignal program, Pcgene software) gives the best score after C_{24}, conforming to the (−3,−1) rule proposed by Von Heijne [34]. It is followed by a prosequence, characteristic of APs of vertebrate, fungal and plant origin [1]. *VuAP1* contains a sequence specific to plant APs referred to as 'PSS' (plant-specific sequence) [1]. The two active site aspartic acid residues, one with the Asp-Thr-Gly motif and the other Asp-Ser-Gly, are consistent with those previously reported [3]. Two putative *N*-glycosylation sites were predicted (Pcgene, Prosite program, Intelligenetics) [35].

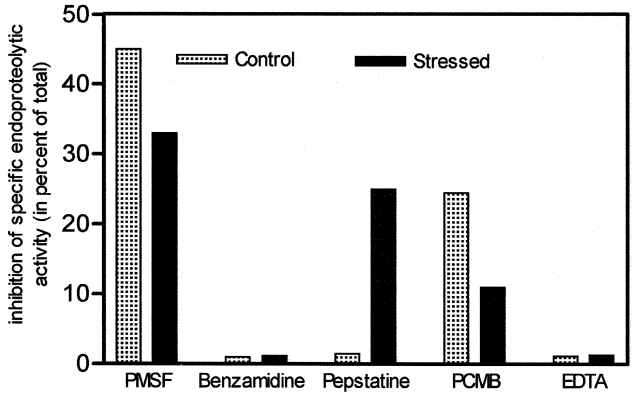

Fig. 2. Effect of class-specific protease inhibitors on endoproteolytic specific activity in partially purified leaf extracts from control and stressed *P. vulgaris* Carioca plants. Control plants: C, $\psi_w = -0.3$ MPa and water-stressed plants: S_2, $\psi_w = -1.5$ MPa. PMSF (serine protease inhibitor), pepstatin A (specific AP inhibitor), PCMB (cysteine protease inhibitor), EDTA (metalloprotease inhibitor). Values are means of three replicates from a representative experiment.

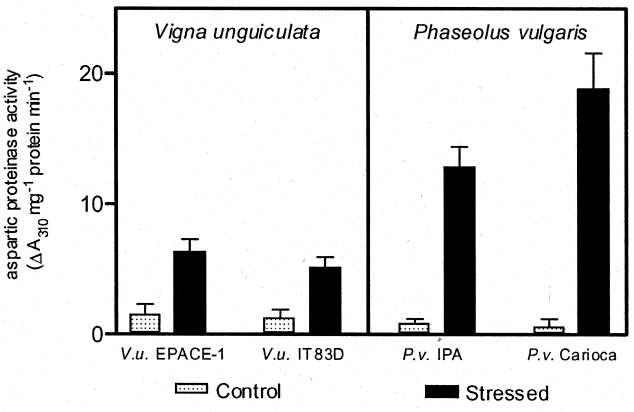

Fig. 3. AP activities in *V. unguiculata* and *P. vulgaris* crude leaf extracts (CE). A specific substrate Pro-Thr-Glu-Phe-(NO_2-Phe)-Arg-Leu was used. The specific AP activity was expressed as ΔA_{310} (mg protein)$^{-1}$ min^{-1}. The leaf extracts were obtained from control (C: $\psi_w = -0.3$ MPa) and water-stressed (S_2: $\psi_w = -1.5$ MPa) *Phaseolus* and *Vigna* plants. Values are means of three replicates from a representative experiment.

Genomic hybridization patterns (data not shown) suggested that the AP precursor gene is encoded by two or more genes in bean, and a single or two genes in cowpea genomes.

3.5. Expression of VuAP1 mRNA under water deficit and ABA treatment

The effects of three levels of water deficit, as well as re-hydration (R), were examined in leaves of bean cv. Carioca and IPA and cowpea cv. EPACE-1 and IT83D. Additional ABA treatment (A+) with control (A−) was carried out using detached leaves. Hybridization was done with a 330 bp long probe corresponding to the 5′ region of the *VuAP1* cDNA.

In bean as well as in cowpea, one band of approximately 1840 bp was detected (Fig. 4). In the case of bean cultivars, transcripts were not detectable in control or in re-hydrated leaves, but appeared under water stress. In IPA the transcript accumulation was similar in S_1, S_2 and S_3 stressed plants while in drought-susceptible Carioca the transcript level increased with increasing stress. The *VuAP1* gene showed no sign of being under the control of ABA in bean leaves under our experimental conditions. In the case of cowpea cultivars, a constitutive expression of transcripts was observed in response to drought stress, re-hydration and ABA treatment on detached leaves.

4. Discussion

Although levels of proteolytic activity are known to be

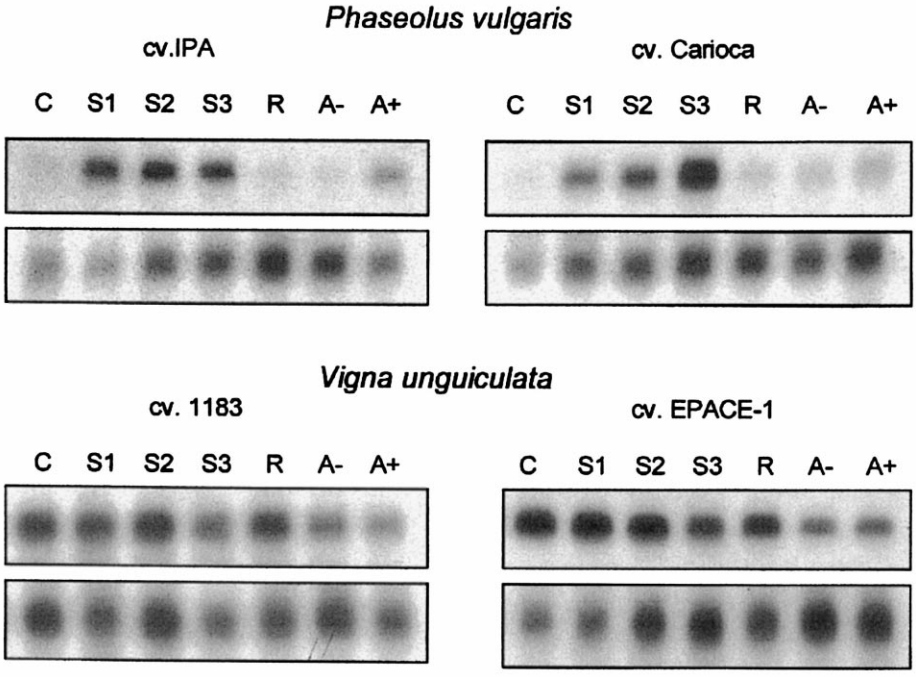

Fig. 4. Northern blot analysis of mRNA of *VuAP1* (A) under water stress, re-hydration and ABA treatment in *P. vulgaris* and *V. unguiculata* leaves. The membranes were hybridized with a ^{32}P-labeled 330 bp fragment of *VuAP1* cDNA under high (*V. unguiculata*) and low (*P. vulgaris*) stringency and with a constitutive mRNA, *nt*S19 probe (B) encoding a constitutively expressed gene of ribosomal protein from *N. tabacum*. C, control, $\psi_w = -0.3$ MPa; S, water-stressed: S_1: $\psi_w = -1$ MPa; S_2: $\psi_w = -1.5$ MPa; S_3: $\psi_w = -2$ MPa; R: re-hydrated after a 24 h water deficit ($\psi_w = -0.3$ MPa). For ABA treatment detached leaves were plunged in 0.1 mM ABA for 24 h (A+) or water (A−).

affected during senescence [36,37], the influence of drought stress is poorly characterized and the relation between plant resistance or susceptibility to drought and protein breakdown has never been established. Using a plant system of four cultivars of common bean and cowpea which differ in their resistance to water stress, we have demonstrated in this study that water deficit induced an increase in endoproteolytic activity that parallels the susceptibility to drought of the cultivar (Fig. 1). Among the different protease classes, cysteine proteinases were shown to be implicated in proteolysis during senescence [38] and under drought [39,40], involvement of APs in senescence of flower petals [12,41] and degradation of pathogen-related proteins [6] was also reported. In this work, the use of class-specific inhibitors has enabled the water stress-stimulated activity in bean Carioca cultivars to be identified as being mainly due to AP (Fig. 2). The highest stimulated specific AP activity was obtained in the case of the more susceptible bean cultivar (Carioca, Fig. 3).

Screening a cDNA library from cowpea leaves led to isolation of a full length cDNA referred to as *VuAP1*, encoding a putative AP precursor showing a high level of similarity to cDNAs of other plant APs. Analysis of the deduced *VuAP1* amino acid sequence showed a PSS of about 100 amino acids not found in yeast or mammalian enzyme homologues. These residues are positioned in the same regions as in other plant AP precursors. The PSS sequence is very similar to that of saposin, a sphingolipid-activating protein from mammalian cells which is involved in targeting proteins to lysosomes [42], and has been suggested to be a vacuolar targeting determinant [43]. It may target proteins to a newly characterized lytic compartment of plant cells called α-TIP PSV [44]. Recent experiments with recombinant cyprosin expressed in *Pichia pastoris* showed that PSS is essential for the correct folding of the protein [45].

Northern and Western experiments showed that the dramatic increase of AP enzymatic activity in bean cultivars correlated with the stimulation of gene expression (Fig. 4) and increased the content of a 36 kDa putative mature AP enzyme form (data not shown). On the contrary, in cowpea cultivars the level of transcripts (Fig. 4) as well as that of AP precursor protein (data not shown) remained unchanged. The observed change in enzymatic activity induced by drought in this species could therefore be due to posttranslational modifications of the immature enzyme form (zymogen processing/activation [1]).

It is interesting to underline that the drought-susceptible and the drought-tolerant plants display different stimulation of AP activity. The capacity of the drought-tolerant cowpea cultivars to maintain enzyme stability under water stress conditions could result from their ability to retain water in the protoplasm [16]. These plants are also able to regulate AP enzyme activity at the level of gene expression (Fig. 4). On the contrary, in susceptible common bean plants, drought induced excessive AP activity (Fig. 3) which probably leads to the deregulation of the balance between catabolism and anabolism. In these susceptible bean plants, a rapid reduction of CO_2 assimilation occurred during water stress [16,17]. Inhibition of photosynthesis may lead to nitrogen starvation during prolonged water stress. The observed enhanced AP activity in droughted leaves could be involved in the proteolytic process of organic nitrogen remobilization to other parts of the plant, notably to the reproductive organs. Plant species as common bean and tomato respond to extended periods of drought with premature flowering and fruit production [46]

and nitrogen remobilization [47]. The successful engineering of plants with the cloned AP precursor *VuAP1* cDNA could help to enlighten the role of this enzyme in plant response to drought stress.

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