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## Variation in relative water content, proline accumulation and stress gene expression in two cowpea landraces under drought

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### Abstract

Many landraces of cowpea [*Vigna unguiculata* (L.) Walp.] are adapted to particular geographical and climatic conditions. Here we describe two landraces grown respectively in arid and temperate areas of Algeria and assess their physiological and molecular responses to drought stress. As expected, when deprived of water cowpea plants lose water over time with a gradual reduction in transpiration rate. The landraces differed in their relative water content (RWC) and whole plant transpiration rate. The landrace from Menia, an arid area, retained more water in adult leaves. Both landraces responded to drought stress at the molecular level by increasing expression of stress-related genes in aerial parts, including proline metabolism genes. Expression of gene(s) encoding proline synthesis enzyme P5CS was up regulated and gene expression of ProDH, a proline catabolism enzyme, was down regulated. Relatively low amounts of proline accumulated in adult leaves with slight differences between the two landraces. During drought stress the most apical part of plants stayed relatively turgid with a high RWC compared to distal parts that wilted. Expression of key stress genes was higher and more proline accumulated at the apex than in distal leaves indicating that cowpea has a non-uniform stress response at the whole plant level. Our study reveals a developmental control of water stress through preferential proline accumulation in the upper tier of the cowpea plant. We also conclude that cowpea landraces display

physiological adaptations to water stress suited to the arid and temperate climates in which they are cultivated.

**Keywords:** Cowpea, drought stress, proline, transpiration, gene expression

## Introduction

Cowpea, the domesticated subspecies of *Vigna unguiculata* [*Vigna unguiculata* (L.) Walp.], is an important crop in semi-arid regions (Pasquet, 1993)(Pasquet, 1999)(Ba et al., 2004). Cowpea originates from the wild *V. unguiculata spontanea* and within the *V. unguiculata* subspecies, cultivated genotypes have been distinguished based on anatomical characteristics, and isozyme and DNA markers (Pasquet, 1993)(Pasquet, 1999)(Ba et al., 2004). Hundreds of cowpea genotypes currently cultivated worldwide are curated in seed banks (Egbadzor et al., 2014)(Ba et al., 2004).

African cowpea landrace diversity has been assessed using anatomical and developmental characteristics and molecular genotyping (Egbadzor et al., 2014)(Ghalmi et al., 2009). Genetically close African cowpea landraces (20 Algerian or 113 Ghana) often have similar features including seed-coat colour, ranging from black to red, mottled or cream (Egbadzor et al., 2014)(Ghalmi et al., 2010). However, geographically and genetically distant landraces with different adaptations may have similar features, for example, by having the same seed-coat colour (Ghalmi et al., 2009)(Muchero et al., 2009)(Huynh et al., 2016).

Compared to other leguminous plants, cowpea is considered to be drought resistant (Belko et al., 2013). Yield, photosynthesis, water content, transpiration and water potential of local cowpea landraces have been compared showing that the ability to withstand drought varies according to the genotype (Singh et al., 2010; Singh and Raja Reddy, 2011). A subset of especially drought resistant cowpea landraces have been identified as possible progenitors for breeding improved cowpea varieties (Hall, 2012).

When cowpea responds to drought stress stomata close rapidly and leaf transpiration is reduced (Hall and Schulze, 1980)(Belko et al., 2013). ABA and lipid signalling and lipid enzyme activity have been correlated to stomatal closure and drought resistance in cowpea (Iuchi et al., 2000)(Torres-Franklin et al., 2007)(Singh and Raja Reddy, 2011). Stress and ABA also induce the expression of stress-related transcription factors, such as DREB2A, which in turn induce a variety of stress-responsive genes (Sadhukhan et al., 2014a).

During water stress, plant mitochondria and chloroplasts accumulate reactive oxygen species (ROS) as by-products of respiration and photosynthesis respectively (Mittler, 2002). A variety of enzymatic or non-enzymatic mechanisms or the induction of alternative electron transport may limit the amount of ROS that accumulates during stress. For example, mitochondrial alternative oxidase (AOX) isoforms can be induced by drought in plants, including cowpea, to limit mitochondrial ROS production (Vanlerberghe et al., 2016)(Costa et al., 2010)(Wang and Vanlerberghe, 2013). The plastid terminal oxidase PTOX, a chloroplast homologue of AOX, is induced in some plant species to limit excess electron transfer in the chloroplast during stress (Laureau et al., 2013) (Johnson and Stepien, 2016).

Another characteristic of stress response in plants is the synthesis of osmolytes, small molecules, such as sugar and the amino acid proline (Szabados and Saviouré, 2010). These molecules allow dehydrated plant cells to withstand dehydration by maintaining turgor, buffering against ROS and maintaining redox homeostasis (Szabados and Saviouré, 2010).

Proline accumulates when it is synthesised from glutamate at the same time as its catabolism is repressed. Drought stress induces expression of the biosynthetic gene *P5CS*, which encodes bi-functional pyrroline-5-carboxylate synthase (EC 2.7.2.11/1.2.1.41) (Pérez-Arellano et al., 2010), and represses *ProDH*, which encodes proline dehydrogenase (EC 1.5.5.2) (Peng et al., 1996) (Verslues and Sharma, 2010). Different signals induce proline biosynthesis in plants. ABA signalling induces *P5CS* (Saviouré et al., 1997)(Strizhov et al., 1997) and lipid signalling, through the action of phosphatidic acid and ROS produced by NADPH oxidases, can modulate *P5CS* and *ProDH* gene expression (Ghars et al., 2012)(Ben Rejeb et al., 2015).

To better characterise cowpea adaptation, we measured variations of key physiological indicators and gene expression in response to drought stress in two cowpea landraces originating from regions of Algeria with either temperate or arid climates. Relative water content (RWC) was measured to better characterize water deficit. The landrace originating from the arid area maintained a higher RWC over the duration of the drought stress and transpired less than the landrace from the temperate area. In both landraces, proline biosynthesis genes are regulated by drought stress but surprisingly, more proline accumulates in the upper tier of the plant compared to older leaves suggesting developmental control of water stress responses.



## Materials and methods

### Plant material and growth

Cowpea [*Vigna unguiculata* (L.) Walp.] landraces were provided by local Algerian farmers. Landrace Bejaia (named B in this work) is grown in Bejaia in north-east Algeria, an area with a sub-humid climate. Landrace Menia (named M in this work) is grown in Menia in central Algeria, a relatively hot and arid region (according to Emberger classification) (Emberger, 1955) (Peel et al., 2007) (Table 1).

Seeds of landrace Bejaia (B) are black, while seeds from landrace Menia (M) are pale or deep golden in colour (Supp. Fig. 1A). The mean 100-seed weight is 7.06 g for Landrace B and 6.91 g for landrace M ( $p < 0.001$  using a Mann-Whitney test with  $n = 10$  samples) (Supp. Fig.1B).

Seeds were surface sterilized, imbibed on moist filter paper overnight at 4°C, and left to germinate at room temperature. Germinated seeds were transplanted into 1800 cm<sup>3</sup> plastic containers containing 60 g of a peat-perlite-vermiculite mixture (2:1:1, v/v/v). The peat (reference V1 mix, Jiffy Products International BV, The Netherlands) is a mix of white and black peat with added fertilizer NFU42001 giving NPK content of 17, 10 and 4 kg/m<sup>3</sup> respectively.

Plants were grown at 22°C in long-day conditions (16h light) in a temperate glasshouse under natural light supplemented with sodium lamps with photosynthetically active radiation (PAR) averaging 90  $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$ . Total PAR was estimated between 90 to 500  $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$ , a makeshift shutter system cutting-off excess light.

### Imposition of drought stress, measuring relative water content and transpiration

Prior to drought the experiment, plants were grown for seven days until the first trifoliate leaves (TFL1) had fully developed, at which time a watering of the soil to saturation was conducted. To impose drought stress, watering was stopped. Relative water content (RWC) was determined for TFL1 or for the upper tier of plants (youngest emerging leaf and apical bud) as stated. The organs were first weighed to determine the fresh weight (FW) then weighed again to determine the turgid weight (TW) after they had been fully hydrated in deionised water for 1 day at 4°C in the dark. The organs were then dried for 2 to 4 days in an oven at 70°C and weighed again to determine the dry weight (DW). RWC was calculated as  $100 \times (\text{FW} - \text{DW})/(\text{TW} - \text{DW})$ . Leaf transpiration was measured using a customised computer-linked weighing machine. After detaching a leaf, the cut petiole was sealed with

grease, and weight loss ascribed to transpiration was recorded for 140 to 180 minutes. Whole plant transpiration was estimated by recording whole plant weight loss after masking the soil surface with plastic film to limit evaporation.

To measure the humidity of the soil, soil was collected from plant pots and weighed (FW). The soil was fully hydrated by soaking it in water then allowing it to drip for 24 hours before weighing (SW). Then the soil was baked to dry it and weighed again (DW). The relative humidity was calculated as a percentage of the fully hydrated soil weight as  $100 \times (FW-DW)/SW$ .

## **Molecular biology**

### **RNA extraction**

For each sample, 100 mg of leaves were homogenized in RNA extraction buffer (0.2 M Tris-HCl pH 7.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS) and mixed with an equal volume of citrate-buffered (pH 4) phenol-chloroform (1:1, v/v). Samples were centrifuged at 10000 rpm for 5 min. The upper aqueous phase was removed and re-extracted with the phenol-chloroform mix. RNA was selectively precipitated from the aqueous phase with 2 M LiCl at 0°C overnight. RNA was pelleted by centrifugation at 12000 rpm for 15 min and dissolved in water. A second precipitation of RNA with 2M LiCl was done for 6 h at 0°C. After centrifugation, RNA pellets were washed with 70% ethanol, dried and dissolved in 50 µL water. RNA was quantified by measuring UV absorbance at 260 and 280 nm using a Nanovue spectrophotometer. After DNase treatment, RNA integrity was checked by electrophoresis on a 1% agarose gel.

### **Semi-quantitative RT-PCR**

For reverse transcription 1.5 µg of RNA was used with Revert Aid Reverse Transcriptase according to the manufacturer's instructions (Thermo Scientific). Complementary DNA samples were diluted 4 fold with ultrapure water. PCR was done using Dream Taq Green DNA polymerase (Thermo Scientific). For each PCR reaction, 2 µL of cDNA was used as a template, 0.8 µM of both forward and reverse primers, 0.2 mM dNTP and 1 unit of DreamTaq in 1 × GreenTaq Buffer. PCR conditions were: 5 min at 94°C, followed by 28 cycles of 30s at 94°C, 30s at 55°C and 30s at 72°C, and finally 10 min at 72°C. In these conditions, the amount of PCR product amplified allows semi-quantitative estimation of the level of expression of each gene tested compared to control gene expression.

### **Oligonucleotide primer design**

Gene sequences were retrieved from the Genespace Sequence (GSS) database of the Cowpea Genomics Initiative (CGKB) (Chen et al. 2007) and from NCBI Genbank. The gene VuEF1, whose expression remains steady during drought stress, was selected as a control. Oligonucleotide primers used for PCR experiments were designed specifically for this study using Primer3 software to have an annealing temperature of 55°C and amplify 200 to 400 bp of cDNA sequence. Table 2 lists the primer sequences of the genes studied with the corresponding Genebank identification numbers and publication reference. Some primers were also designed and described in Y. Maia (2012).

#### **Proline and MDA quantification**

Proline content was determined by a colorimetric method adapted from Bates et al., 1973. Pulverized frozen tissue (20 to 50 mg) was homogenized in 0.75 mL of 3% sulfosalicylic acid. The homogenate was centrifuged at 14000 rpm at 4°C for 10 min. Ninhydrin buffer (2.5% ninhydrin, 60% acetic acid in 2.5M phosphoric acid) and 100% acetic acid were added to 0.4 mL of supernatant (1:1:1, v/v/v). The colour reaction was developed for 60 min at 95°C. After cooling, 0.8 mL of toluene was added to each sample. The optical density of the upper organic phase was read at 520 nm. Proline was quantified by comparison with L-proline as the standard. MDA was quantified using the thiobarbituric acid (TBA) colorimetric reaction according to Hodges et al. (1999).



## Results

### Dynamics of water loss are different in two cowpea landraces from Algeria

Two cowpea landraces originating distant areas of Algeria, were expected to be genetically and physiologically adapted to their local climates or environments. Bejaia has a sub-humid climate, while Menia is an arid area (according to Emberger classification) (Emberger, 1955) (Peel et al., 2007) (see section 2.1, Table 1).

We wanted to test the molecular and physiological response of cowpea to progressive dehydration, which mimics natural drought conditions better than hyperosmotic stress, as can result in severe dehydration. The RWC of the first trifoliate leaves (TFL1) of each landrace was measured during two weeks of progressive dehydration. At the start, the RWC of both cowpea genotypes was above 95% (Figure 1). After one week without water RWC lowers, however, landrace M had a higher RWC than B (Fig. 1A). This difference widened over time. For example, after 13 d landrace M had 72% RWC compared to 57% for landrace B. The difference in TFL1 RWC was even greater after 15 d when landrace B had lost 30% more water than landrace M (Fig. 1A). The humidity of the soil was confirmed to be similar for both M and B samples, diminishing over time at a similar rate (Fig. 1B). We can therefore hypothesize that when subjected to water stress the landrace from the arid region is able to retain water more effectively than the landrace from a sub-humid area. However, RWC values at the end of the stress experiment are much lower than usually measured in field studies and might be unrepresentative of natural field conditions (Cruz de carvalho et al., 1998, Belko et al., 2013).

### Leaf and whole plant transpiration

We investigated whether the difference in RWC between the cowpea landraces is related to leaf and whole plant transpiration. We measured water loss from detached TFL1 from M and B plants with similar RWC (Figure 2A-C). When plants are fully hydrated their transpiration rate is maximal with evaporation of 10 g of water in 1 h (Figure 2A). The transpiration rate was threefold less in leaves from partially dehydrated plants with RWC of 85-87% (Fig. 2B). Transpiration is ten times less than the maximum in plants with 76-78% RWC with 1 g of water lost per h (Fig. 2C). TFL1 transpiration rates for the two landraces were similar for plants with similar RWC. TFL1 area is slightly but not significantly different between landraces (Landrace Bejaia 39.25 cm<sup>2</sup> +/- 5.15; landrace Menia 34.66 +/- 5.57).

Plants had similar total leaf area (73.5 cm<sup>2</sup> for Bejaia and 76.26 cm<sup>2</sup> for Menia) Whole plant transpiration was recorded in dehydrating plants. (Figure 3A, B). For 3 d after watering soil to saturation, transpiration increased to similar high rates for both landraces (Fig. 3A). After 7 d of water deprivation, daily transpiration is reduced by 80% (Fig. 3B). From this point, daily water loss was always higher for the B landrace (Fig. 3B). However, variations within samples were too large for this difference to be highly statistically significant ( $p > 0.05$  using a Mann-Whitney test). While single leaf transpiration is similar in the two cowpea landraces the kinetics of whole plant transpiration could be slightly different.

### **Progressive drought triggers expression of stress-response and proline-synthesis genes but little proline accumulation**

When plants undergo water stress, specific changes in expression of key genes occur that in turn modulate other genes to generate a physiological response (Shinozaki and Yamaguchi-Shinozaki, 1997)(Bray, 2004). *VuDREB2A*, which encodes DREB2A transcription factor, and *VuNCED1*, which encodes NCED1 enzyme involved in ABA synthesis, have both been characterised in cowpea (Sadhukhan et al., 2014)(Iuchi et al., 2000). *VuDREB2A* and *VuNCED1* mRNA levels increased as early as 7 d after the onset of water deprivation which corresponded to 88% RWC for the M landrace and 82% RWC for the B landrace. (Sadhukhan et al., 2014)(Iuchi et al., 2000) mRNA levels remained high during the second week of dehydration fluctuating slightly (Figure 4). We tested the effect of water stress on chloroplast and mitochondria electron transfer which can result in oxidative stress (Bartoli et al., 2005). Expression of the chloroplast PTOX gene is part of the stress response of some plant species (Johnson and Stepien, 2016)(Laureau et al., 2013). A low level of PTOX expression was detected in cowpea in control conditions (Fig. 4A). *VuPTOX* mRNA accumulated during water deprivation and remained at this higher level (Fig. 4A). Not all genes encoding chloroplast functions are up regulated. For example, expression of *VuRBCS*, encoding the Rubisco small subunit, did not vary (Fig. 4A). We also tested mitochondrial AOX expression. Some, but not all, AOX genes are up-regulated during stresses that induce mitochondrial dysfunction or during oxidative stress (Costa et al., 2010)(Vanlerberghe et al., 2016). In our experiment, *VuAOX 1* and *2b* mRNA accumulation did not vary in either landrace (Fig. 4A). Malonyl dihydroxyacetate (MDA), a marker of oxidative stress, resulting from lipid peroxidation (Hodges et al., 1999) was tested (Supplementary Figure 2). MDA increased 39% in landrace B and 75% in landrace M after 15 days of drought suggesting a

oxidative stress and a lipid peroxidation as a consequence of draught stress more pronounced in the B landrace (Supplementary Figure 2).

Proline metabolism genes are targets of stress regulation in cowpea (Shui et al., 2013). The *P5CS* gene encodes the enzyme responsible for the rate-limiting step of proline synthesis. We found that the proline biosynthesis *VuP5CS* mRNA was barely detectable in control conditions but it accumulated as early as 7 d after the start of dehydration (Fig. 4A). More *VuP5CS* mRNA accumulated as RWC decreased. However, even though RWC of the B landrace decreased more, the *VuP5CS* mRNA signal was not higher than in the M landrace (Fig. 4A). *VuProDH* mRNA encoding the proline catabolism enzyme ProDH was detected in cowpea in the control conditions but levels decreased during stress (Fig. 5A). After 7 d of water deprivation landrace M had higher *VuProDH* mRNA levels and higher RWC than landrace B (Fig. 4A).

As cowpea *P5CS* and *ProDH* were regulated by water stress at the gene expression level, proline synthesis could also have been up-regulated and proline catabolism down-regulated. To test this, we tried to correlate gene expression and proline accumulation in TFL1 in response to water stress. There was a lag of several days between induction of *P5CS* gene expression and the accumulation of measurable amounts of proline. Proline was barely detectable for 10 d after the onset of the water deprivation although *VuP5CS* mRNA had increased after 7 d (Fig. 4A-B). A difference in proline accumulation between landraces became noticeable after 13 d. Landrace B accumulated up to 300 nmol of proline per g of DW, 5 times more than landrace M (Fig. 4B), which also correlates with the difference in RWC between genotypes.

### **Gene expressions and proline accumulation in younger leaves**

We observed that the upper tier of cowpea plants, including the apex and younger leaves, was more turgid than TFL1 after two weeks of dehydration (Supplementary Figure 3). RWC of upper tiers remained above 65% for the whole dehydration period in both landraces (Figure 5). We investigated the gene expression response to water stress in upper tiers (Fig. 5A). Both *VuDREB2A* and *VuP5CS* were expressed in the absence of stress and up-regulated as RWC decreased (Fig. 5A). *VuProDH* expression was down regulated upon stress. When plants were watered again after deprivation, *VuP5CS* and *VuDREB2A* levels decreased while *VuPRODH* levels increased. Stress and stress relief were therefore readily perceived by tissues at the apex, even though the variation in RWC was limited (Fig. 5A).

Proline content of the upper tier was very low in the absence of stress (Fig. 5B). Proline content increased 20 to 30 fold with stress, reaching 45  $\mu\text{mol}$  per g of DW when RWC was the lowest after 2 weeks of dehydration. Both cowpea genotypes had accumulated similar amounts of proline at similar RWC. Most strikingly, more proline accumulated in the upper tier than in TFL1. From 0.306 ( $\mu\text{mol}$  /g, DW) proline in TFL1 to 49.6  $\mu\text{mol}$  proline in upper tier, proline is 100 times more abundant in landrace B upper tier compared to TFL1. Similarly, for landrace M, from 0.047 ( $\mu\text{mol}$  /g, DW) proline in TFL1 to 44.82 in upper tier, proline is 900 times more abundant in landrace M in upper tier compared to TFL1. (Fig. 5B).

## Discussion

### RWC variation and transpiration in cowpea

Here we describe two cowpea landraces from Algeria. The landraces are easily distinguished by their different seed colours, which might be due to mutations in flavonoid biosynthesis genes occurring independently in local populations, as seen for example in the *Glycine max* (soybean) chalcone synthase gene (Todd and Vodkin, 1996)(Tuteja et al., 2009). Seed colour may indicate how closely related two landraces are but is not a sufficient marker of diversity on its own.

Cowpea plants regulate water loss according to whether they are well watered or subject to drought or vapour pressure deficiency (Hall and Schulze, 1980). The basis of this regulation has been ascribed to soil-root interaction and whole plant signalling rather than to a decline in leaf water potential (Osonubi, 1985). Landrace M, which originates from an area with an arid climate, maintained a higher RWC than landrace B over the two-week dehydration. Other comparisons of cultivated varieties or wild populations have shown that RWC can be used to distinguish between varieties that are tolerant or susceptible to drought. For example, comparison of RWC amongst drought tolerant and sensitive beans (*Phaseolus vulgaris*) allowed ecotypes to be distinguished (Rosales et al., 2011). In the model plant *Arabidopsis*, different ecotypes (or accessions) can be distinguished based on variation in RWC in response to mild drought. Plant populations adapted to arid climates can also be distinguished by direct measurement of RWC or indirectly by measuring the ratio of carbon isotope delta C13 (McKay et al., 2003). There is likely to be a range of physiological adaptations as RWC itself varies amongst *Arabidopsis* ecotypes in well-watered conditions

and under drought (Bac-Molenaar et al., 2016). Midday RWC drop is a parameter that allows discrimination between some cowpea ecotypes (Kumar et al., 2008; Hayatu et al., 2014).

The two landraces had an almost identical RWC over the first week of drought stress. This is contrary to what has previously been observed in well-watered plants from arid and sub-humid climates. Only after 8 days did RWC differ in landrace B and M. It is therefore possible that the landraces are both tolerant to mild drought stress, that can only be distinguished in terms of RWC after severe water shortage.

Transpiration was nearly identical in both landraces when well-watered. Our results show that leaf transpiration from isolated TFL1 varies when plants are subjected to drought. A threefold decrease in transpiration is observed after one week of stress when RWC is only moderately lower (85-87% compared to 93%), illustrating the tight regulation of cowpea transpiration by stomatal closure (Belko et al., 2013). Furthermore, in stressed plants with 76-78% RWC, transpiration was tenfold less. RWC of landrace M remains higher for longer than in the B landrace so total transpiration from TFL1 is potentially greater in the M landrace, an apparently paradoxical observation. However, all transpiration rates are very low and TFL1 might not represent whole plant transpiration.

Whole plant transpiration of well-watered plants is mostly similar between landraces. In landrace M, however, transpiration is slightly lower during drought. Several factors, such as ABA, CO<sub>2</sub> and redox status, contribute to stomata closure and transpiration regulation during drought (Kim et al., 2010)(Djebbar et al., 2012)(Singh and Raja Reddy, 2011). For example, in cowpea plants that were dehydrated for 10 h after uprooting, endogenous ABA increased 140 times in leaves (Iuchi et al., 1996)(Iuchi et al., 2000). Studying 40 cowpea genotypes, Belko et al. (2013) found that drought-sensitive genotypes had higher transpiration rates than drought-tolerant genotypes both in well-watered and vapour pressure deficit conditions (Belko et al., 2013). Natural variation in drought resistance in a related species, *Vigna unguiculata sesquipedalis* (asparagus bean), was also attributed to variation in transpiration rates in response to vapour pressure deficit and soil drought (Xu et al., 2015). An explanation for our results could be that transpiration physiology and other mechanisms might contribute to local adaptation. Five quantitative trait loci related to drought and senescence physiology have been identified by studying the natural variation in local cowpea landraces, including the “stay green” phenotype (Muchero et al., 2009)(Muchero et al., 2010). Similar quantitative loci were also identified in asparagus bean

(Xu et al., 2015). Whole plant physiology mechanisms therefore contribute to plant drought-stress adaptation.

### **Stress response revealed by gene expression**

Genes expressed in cowpea in response to drought stress include *VuDREB2A*, whose gene product is similar to Arabidopsis *DREB2A* transcription factor that regulates other genes involved in the molecular stress response including ABA biosynthetic genes (Sadhukhan et al., 2014a)(Sadhukhan et al., 2014b). *VuDREB2A* and *VuNCEDI* expression were previously found to be induced 6 h after the onset of dehydration and prior to ABA accumulation in stems and leaves, but not in roots (Iuchi et al., 2000)(Sadhukhan et al., 2014a). We found *VuNCEDI* expression increased in both landraces during drought stress with a stronger signal in landrace B after 13 d. The latter observation could be a consequence of landrace B having a lower RWC at this stage. Although the results suggest that cowpea responds to dehydration by modulating ABA synthesis, the stronger expression of *VuNCEDI* in landrace B did not lead to better control of transpiration.

Membrane lipid metabolism and antioxidant genes are induced by drought stress in cowpea (Torres-Franklin et al., 2007, 2008). Stress-regulation of mitochondrial AOX is well documented (Bartoli et al., 2005)(Vanlerberghe et al., 2016). In cowpea seedlings, *VuAOX* genes 1 and 2b (Costa et al., 2007)(Costa et al., 2010) are transiently expressed and AOX activity increases under short severe drought stress (Costa et al., 2010). In the two landraces studied here, the two *VuAOX* mRNA tested were not up-regulated after days of progressive dehydration. It is possible that AOX is activated at the protein level though (Umbach et al., 2006).

Chloroplast PTOX resembles AOX (Carol et al., 1999)(Carol and Kuntz, 2001) and is associated with carotenoid synthesis and chlororespiration (Josse et al., 2000). PTOX is up-regulated by stress in some plant species (Laureau et al., 2013)(Stepien and Johnson, 2009)(Johnson and Stepien, 2016). PTOX electron transfer to dioxygen, bypassing photosynthetic cytochrome B6f and photosystem I, may act as a safety valve for transfer of excess electrons from the photosynthetic electron transfer chain and ROS formation (Laureau et al., 2013)(Krieger-Liszkay and Feilke, 2015)(Nawrocki et al., 2015). We found clear and sustained up-regulation of *PTOX* gene expression in landraces B and M in response to drought. In this respect, cowpea resembles plants adapted to extreme environments such as the alpine plant *Ranunculus glacialis* or the halophyte *Thellungiella halophila* (Laureau et al., 2013)(Stepien and Johnson, 2009).

Proline synthesis and catabolism genes are regulated by several stresses (Peng et al., 1996) to allow the accumulation of proline, a compatible solute with protection properties (Yoshiba et al., 1997)(Szabados and Saviouré, 2010). We observed that *P5CS* was highly induced and *ProDH* repressed in TFL1 in response to drought stress. This suggests proline was synthesised *de novo* in TFL1. Unexpectedly, the B landrace from a temperate climate accumulates more proline in TFL1 than the M landrace from an arid climate, suggesting that proline accumulation in TFL1 does not represent an adaptation. Although fivefold more proline accumulated in the TFL1 of landrace B, this amount of proline was still low, an estimated 30 times less than accumulates in *Arabidopsis* and other plant species in comparable conditions (Szabados and Saviouré, 2010). Proline accumulation is known to vary between genotypes in *Arabidopsis* (Sharma et al., 2013)(Verslues and Sharma, 2010). In leaves of different cowpea genotypes subjected to drought stress, proline content varied from 0.77 to 3.9  $\mu\text{mol}$  per g of FW (Singh and Raja Reddy, 2011)(Costa et al., 2011). We found 10 to 100 times less proline in TFL1. Possibly, other studies used younger leaves or several leaves from the same plant rather than fully grown TFL1 (Singh and Raja Reddy, 2011)(Costa et al., 2011). Because levels of *P5CS* and *PDH* expression in TFL1 did not correlate with the low amount of proline found there, it is possible that another regulatory mechanism prevents proline synthesis or accumulation in TFL1. The contribution of proline accumulation to osmotic adjustment in plants is variable according to the plant species or the organ (Slama et al., 2006, Verslues and Sharma, S., 2010). However, our results suggest proline accumulation might contribute to osmotic adjustment in cowpea younger leaves.

### **Stress perception in plant upper tier**

The RWC of the upper tier decreased during drought stress but remained higher than TFL1 RWC. This has been observed in *Arabidopsis* where RWC of young leaves was higher than in old leaves under drought (Sperdouli and Moustakas, 2014). Stress sensing by the upper tier of the plant was confirmed by the observed changes in expression of *VuP5CS*, *VuProDH* and *VuDREB2A* genes. These genes were expressed prior to the onset of water stress but as RWC decreased, *VuProDH* gene expression was downregulated while *VuP5CS* and *VuDREB2A* gene expression was slightly up-regulated. Interestingly, watering plants at the end of the two-week experiment increases RWC and strongly down-regulated *VuP5CS* and *VuDREB2A*, showing that the apical part of the plant is sensitive to both decrease and increase in RWC. It has been shown that mobile signals including ABA and small RNA are

associated with water-stress in cowpea (Barrera-Figueroa et al., 2011)(Shui et al., 2013). Such signals might contribute to regulating stress-related genes in distant organs.

Proline accumulation and gene expression in TFL1 and the most apical part of the shoot were regulated differently relative to RWC. Proline accumulation was 100 times higher in the upper tier compared to TFL1, suggesting either a more efficient proline synthesis or less catabolism. Younger leaves accumulate more proline than older leaves in *Arabidopsis* under drought stress (Sperdouli and Moustakas, 2014). In soybean after 9 d of drought stress, 7 times more free proline accumulates in young leaves than in older and senescent leaves (Fukutoku and Yamada, 1984). Phloem sap has also been shown to be a source of proline. Drought stressed alfalfa accumulates 60 times more proline in the sap than controls (Girousse et al., 1996), suggesting that proline is transported in the phloem from source leaves to meristematic tissues. Possibly source organs, such as TFL1, contribute to proline accumulation in young sink organs.

### **Conclusion**

We found that two local cowpea landraces chosen from distinct geographical areas in north-east and central Algeria, respectively, respond differently to progressive simulated drought. Landrace M from an arid area retained more water in adult leaves but both landraces retained water in the upper tiers of the plant during dehydration.

Stress-related genes and proline metabolism genes are regulated after the onset of stress when TFL1 RWC was still above 80%. This induction of stress-related genes mirrored the down regulation of transpiration. Despite having gene expression hallmarks of increased proline biosynthesis in TFL1, only a small amount of proline accumulated in TFL1 but large amounts accumulated in the upper tier. Proline accumulation is therefore regulated between source and sink organs in the whole plant similarly in both landraces.

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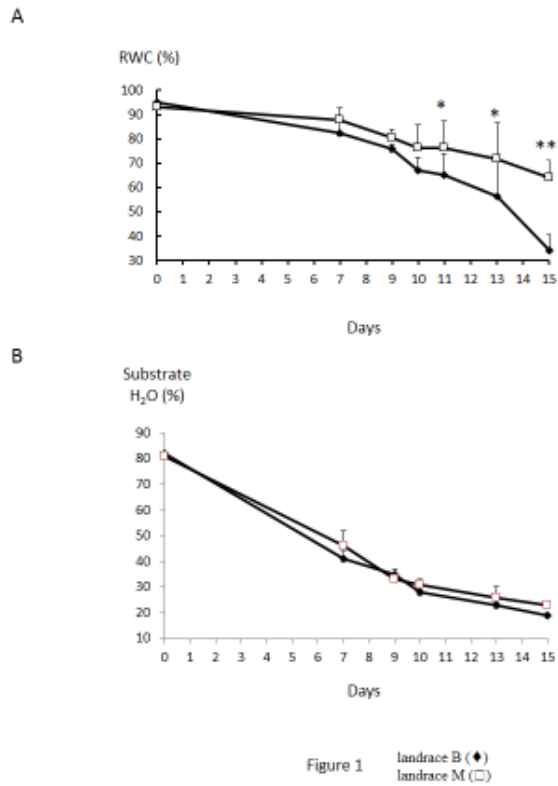
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**Figure 1. Decrease in relative water content in cowpea landraces.**

A, Relative water content (RWC) of first trifoliolate leaves and B, soil humidity during water deprivation of landrace B (◆) and landrace M (□). Measurements were done daily after soil was watered to saturation on Day 0. Significant differences between landraces ( $n = 8$ ) are indicated by \* for  $p < 0.05$  and \*\* for  $p < 0.01$  in Mann-Whitney tests. Error bars represent standard deviation.

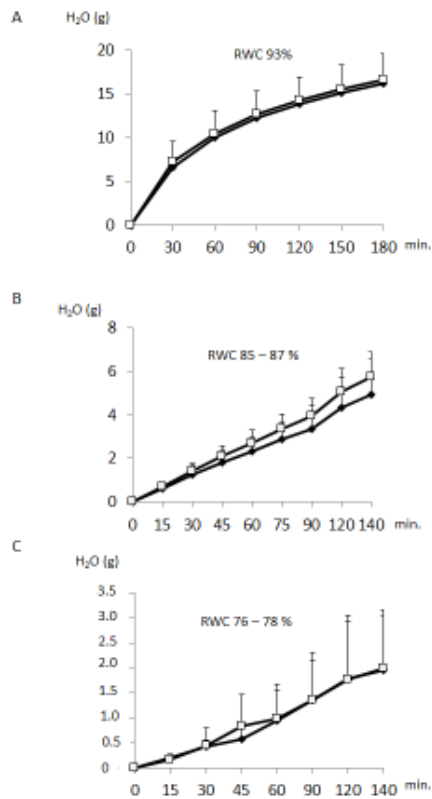
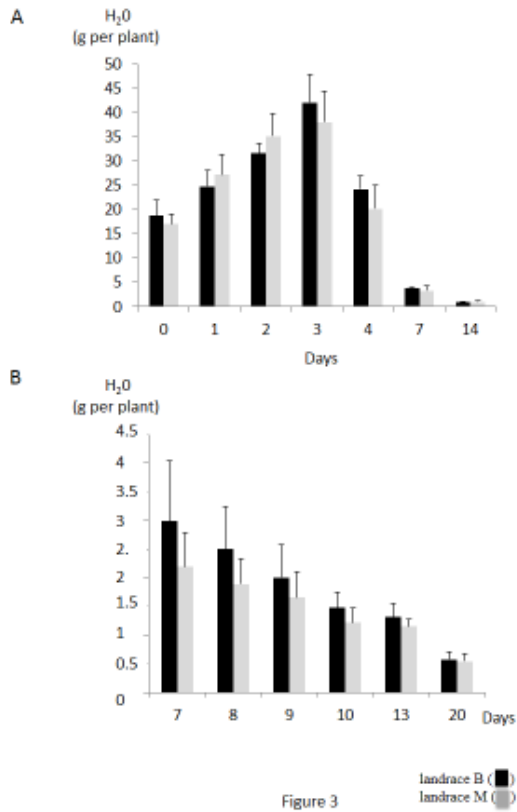
**Figure 2. Leaf transpiration rates decrease during water deprivation.**

Figure 2 landrace B (◆); landrace M (□)

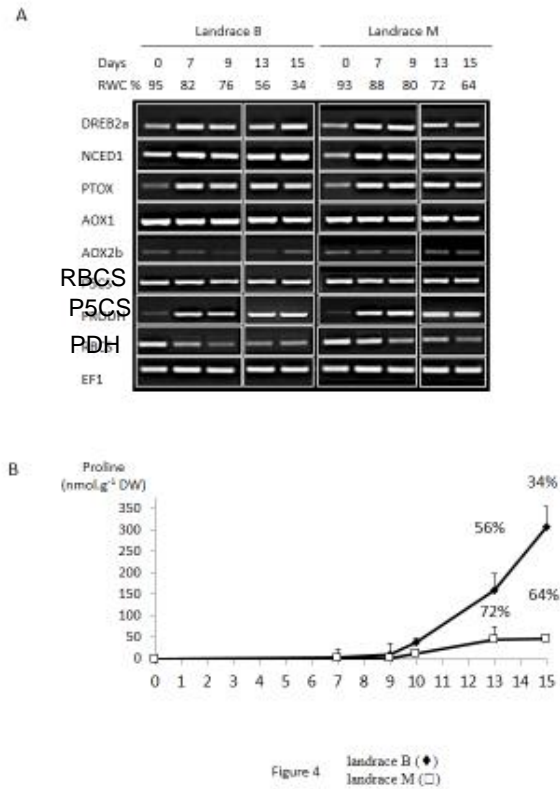
The amount of water transpired by detached TFL1 of landrace B (◆) and landrace M (□) was recorded over 2-3 hours and results are given as a percentage of the initial leaf RWC of A, 93%, B, 85-87% and C, 76-78%. Transpiration rates were significantly different at  $p < 0.01$  in Mann-Whitney tests between the three RWC categories, but there was no difference between landraces at the same RWC. Error bars represent standard deviation.

**Figure 3. Whole plant transpiration rates decrease during water deprivation.**



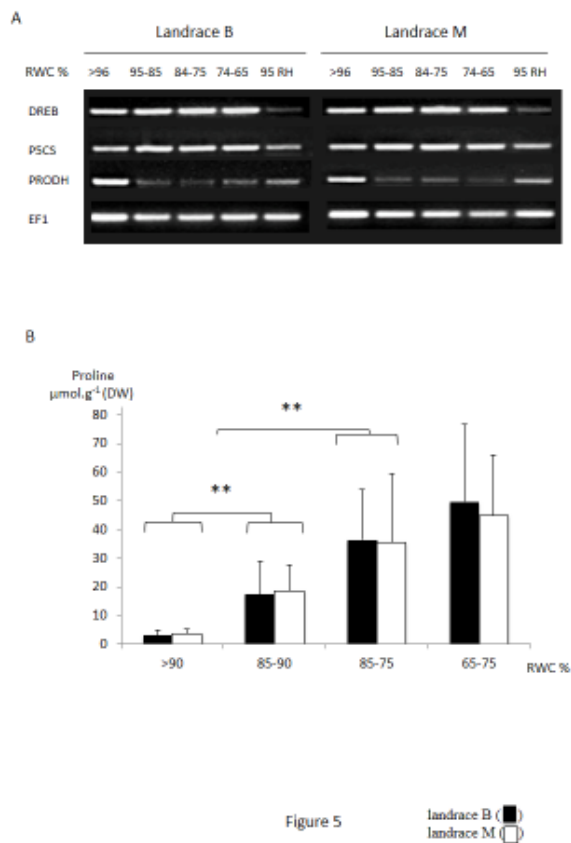
A, Water loss from whole plants was recorded daily for the first week of water deprivation and again after 2 weeks. B, Water loss recorded between 7 and 20 days after the start of water deprivation. Differences between landrace B (black bars) and landrace M (grey bars) were found consistently but are only moderately significant ( $n = 9$  plants,  $p = 0.07$  in Mann-Whitney test).

**Figure 4. Stress-related and proline-metabolism gene expression and proline content in TFL1.**



Number of days after the start of water deprivation and RWC values measured at the time of tissue harvest are indicated. A, RT-PCR was used to test gene expression levels in cowpea landraces using semi-quantitative conditions that reveal differences in the amounts of template molecules without saturation of the amplification reaction. EF1, gene encoding EF1alpha, whose expression is constant, was used as a reference. Details of enzyme activities and biological roles are given in the main text and details of gene and primer sequences are given in Table 2. B, Proline content in TFL1 of landrace B (◆) and landrace M (□) following water deprivation. The values above each curve indicate the corresponding RWC. Differences in proline content between landraces ( $n = 4$ ) are significant after 13 and 15 days with  $p < 0.01$  in a Mann-Whitney test.

**Figure 5. Proline-metabolism gene expression and proline content in upper tiers of cowpea plants during water stress.**



A, RT-PCR was used to test gene expression levels in upper tiers of cowpea landraces using semi-quantitative conditions that reveal differences in the amounts of template molecules without saturation of the amplification reaction. EF1, gene encoding EF1alpha, whose expression is constant, was used as a reference, Details of enzyme activities and biological roles are given in the main text and details of gene and primer sequences are given in Table 2. The percentage values below indicate the corresponding RWC during water deprivation, while 95 RH indicates samples that were watered at the end of dehydration period. B, Proline content in upper tiers of landrace B (black bars) and landrace M (clear bars) deprived of water. RWC values of tissues at the time of harvest are indicated. \*\*, significant differences between samples (n = 6) at  $p < 0.01$  in Mann-Whitney test.

**Table 1: Characteristics of geographical origin of cowpea landraces used in this study.** T°: temperature.

Landrace	Bejaia (B)	Menia (M)
Area of origin	Bejaia (North-East Algeria)	Menia (Central Algeria)
Geographical location	36°45'N, 5°05' E	30°35' N, 2°51' E
Elevation above sea level	86 m	387 m
Climate (Emberger) (Emberger, 1955)	Sub humid	Hot arid
Climate (Köppen-Geiger) (Peel et al., 2007)	Csa	BWh
Average Temperature	17.6 °C	21.9 °C
Annual Rainfall	834mm	34mm

**Table 2: Oligonucleotide DNA primers used in this study**

<b>Gene</b>	<b>Primer F</b>	<b>Primer R</b>	<b>Sequence name, Genbank and publication</b>
<b>AOX1</b>	AAGATGGAGAAGAAGGTTGGT	TGTATGTCTCCCATGGCCTA	<b>Costa et al., 2010</b>
<b>AOX2B</b>	CTTCAAGGAACGGTATGGG	AGAGTGCTGAAACTTCCTC	<b>(Genbank AJ421015) Costa et al., 2010</b>
<b>PTOX</b>	TCCAAATCATCTCCACTTCAC	CTTTATCACAGAATCCGTGAG	<b>Maia, 2012</b>
<b>P5CS</b>	TAACTCACCTCAACTCGCTC	AACAGCACCTGAAGTTACCA	<b><i>VuP5CS</i> (Genbank AB056452.2 )</b>
<b>PDH</b>	ATGCTCGAGAAGATTGCTGA	ACAGGTCCAATGGCATGTA	<b><i>VuProdh</i> (Genbank AB779665) Kaneko et al., 2013</b>
<b>DREB2A</b>	AAGCTGCTCTTGCCATGAT	CTGCTAGTTGGTGAGGCATA	<b>VuDREB2A (Genbank JN629045.3) Sadhukhan et al., 2014</b>
<b>NCED1</b>	GATGCACGATTCGCCATAA	GGTGAAGCAGAAACAATCC	<b><i>VuCPRD65 VuNCED1</i> (AB030293.1) neoxanthine cleavage system Iuchi et al., 2000</b>
<b>EF1a</b>	GTAACAAGATGGATGCCACC	CCACTTTCTTCAAATACGAGGAG	<b>Maia, 2012</b>
<b>RBCS</b>	CATACCGTGAGCAAAACAGG	GGCAATGAAACTGATGCACT	<b><i>VuRBCS</i> (Genbank JN692253.2)</b>