REGULAR ARTICLE

Physiological responses and membrane integrity in three *Vigna* genotypes with contrasting drought tolerance

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Abstract

In the present work some dehydration-induced responses at whole plant and cellular levels were evaluated in three *Vigna* genotypes (*V. glabriscens*, Vg; *V. unguiculata* cv. EPACE-1; *V. unguiculata* cv. 1183) from different origins. Changes in leaf stomatal conductance (gₛ), membrane lipids composition and abscisic acid (ABA) content were determined under water stress. Membrane integrity was assessed through leaf discs dehydration with polyethylene glycol (PEG 8000, -1.3 MPa), and expressed as an injury index (I%). In EPACE-1 and 1183, rapid stomatal closure at early stages of dehydration was related with the ability to accumulate endogenous ABA. On the contrary in Vg stomata closed at lower leaf relative water content as ABA increase was delayed. In 1183, high I% values occurred in PEG-dehydrated discs, and lipid degradation was observed even under moderate water stress (S1), becoming significant under more severe drought (S2). Also, along drought imposition this genotype presented a continuous leaf ABA increase that probably contributed to early visual tissue senescence. Vg and EPACE-1 presented lower I% values, probably denoting the ability to preserve membrane integrity. In EPACE-1 this was achieved through a quite stable lipid content, while in Vg new membrane lipids were synthesized. It is suggested that a higher protoplasmic tolerance in EPACE-1 and Vg might be related to stable and low endogenous ABA contents under increasing water deficit.

Key words: *Vigna*, Abscisic acid, Drought, Lipids, Membrane leakage, Stomatal conductance

Introduction

Legumes are agronomically important crops mainly due to their nitrogen fixing capacity and to their high protein content. *Vigna* species and varieties successfully grow in arid to semi-arid regions, and additionally constitute a valuable genetic resource in studies concerning drought, one of the main factors affecting crop productivity and quality (Van der Maesen and Somaatmadja, 1989).

Cell membranes are major targets of environmental stresses (Lesich et al., 1992) and in *vitro* screening tests for membrane permeability are frequently used to evaluate membrane tolerance to dehydration (Vasquez-Tello et al., 1990; Navari-Izzo et al., 1993; Campos et al., 1997). Altered membrane permeability may result in increased leakage from cells due to the appearance of heterogeneous membrane domains as regards lipid phases and configurations (Lesich, 1992), or to damage of membrane components, namely in the lipid matrix (Harwood, 1998). However there are changes in lipid composition of higher-plant membranes that may contribute to preserve membrane integrity and cell compartmentation (Kuiper, 1985), and hence to control metabolic activity (Bewley, 1993; Harwood, 1998). Such an adaptive role for qualitative and quantitative modifications of membrane lipids has been reported under different environmental stresses, namely high irradiance (Ramalho et al. 1998), cold (Öquist 1982; Partelli et al., 2011), heat (Dias et al., 2010) and drought (Pham Thi et al., 1990; Matos et al., 2010).
On the other hand, plants respond to water deficit by accumulating abscisic acid (ABA) and genetic variation in the capacity to accumulate ABA is known among crop species (Pekic and Quarrie, 1988; Quarrie, 1991; Tuberosa et al., 1994). The rapid translocation of ABA in shoots via xylem flux and the increase of ABA concentration in plant organs correlate with the major physiological changes occurring under water stress conditions, playing a predominant role in drought avoidance through the induction of stomatal closure (Wilkinson and Davies, 2002). Genetic variation in shoot sensitivity to ABA imported from the root may be important in mediating the root signal effect (Blum and Sinmena, 1995). This growth regulator is also involved in triggering tolerance mechanisms at cellular level (Ingram and Bartels, 1996). Direct effect of ABA on membranes has also been reported, using both natural and artificial membrane systems. ABA can directly affect membrane physical properties, like microviscosity (Shripathi et al., 1997), fluidity (Stillwell et al., 1988), and permeability to ions and solutes (Stillwell and Hester, 1984). Some of these modifications seem to be related to changes in lipid composition of cellular membranes (Shripathi et al., 1997; Campos and Pham Thi, 1997).

The studied Vigna genotypes were previously compared as regards the effects of water deficit on photosynthetic performance and water relations (Campos et al., 1999a). The present work deals with whole plant and cellular responses underlying contrasting drought sensitivities in three Vigna genotypes from different origins, focusing the changes in stomatal conductance, ABA content, lipid composition and membrane integrity in leaves under dehydration conditions. Results are expected to contribute for the better understanding of physiological responses and/or constitutive features be relevant in the process of plants adaptation to drought.

Material and Methods

Plant material and experimental conditions

For this study we selected three genotypes belonging to the genus Vigna (Phaseolidae) with contrasting drought sensitivities. V. glabrescens (Vg) is a wild species originating from the Philippines (drought-resistant), V. unguiculata cv. 1183 (drought-sensitive) is cultivated in humid regions of China, and V. unguiculata cv. EPACE-1 (drought-resistant) is grown in the semi-arid North-East of Brazil. Seeds were provided by the University of Gembloux (Belgium) and the Agronomy Research Station of Ceará (Brazil).

After germination, plants were grown in pots, in a mixture of vermiculite: Triohum-Tray substrat (4:5) and were irrigated with two fold micronutrients Hoagland and Snyder solution, as described in Campos et al. (1999a). Plants were kept in a greenhouse under natural irradiance (with a maximal photosynthetic photon flux density of ca. 800-900 μmol m⁻² s⁻¹), mean diurnal temperatures of 30°C and relative humidity values between 70% (morning) and 40% (late afternoon). Dehydration was progressively induced by withholding irrigation for 10 to 12 days in six weeks old plants. According to relative water content (RWC) values, two drought levels were considered: mild stress (S1, RWC 65-75%) and severe stress (S2, RWC < 60%). All determinations were carried out on recently fully expanded leaves.

Plant water status

Relative water content (RWC) was calculated as previously described (Campos et al., 1999a) from samples of 10 leaf discs of 0.5 cm², as RWC=(FW-DW/TW-DW)×100, where FW is the fresh weight, TW is the turgid weight after overnight water saturation of the discs in a humid chamber at room temperature, and DW is the dry weight after drying at 80°C for 24 h.

Stomatal conductance measurements

Stomatal conductance for water vapour (gₛ) was measured under a photosynthetic photon flux density of 1300-1600 μmol m⁻² s⁻¹, in one attached leaf of three plants from each treatment, using a portable IRGA LI-6200 (LI-COR, Inc., Nebraska, USA). Measurements were performed between 10:00 and 11:00 a.m.

Quantification of leaf ABA content

Leaf samples (ca. 400 mg FW) were collected along the dehydration period, frozen in liquid nitrogen, freeze-dried and stored over silica gel in the dark, at ca. 20°C. For ABA determinations, the freeze-dried samples were cut to a fine powder and extracted in deionized water at 5°C overnight. Samples were centrifuged and the supernatant removed for analysis by radioimmunoassay (RIA), following Quarrie et al. (1988). The monoclonal antibody used (AFR MAC 62) is specific for (+)-ABA. Cross-reactivity of the assay with substances other than ABA was tested on Vigna genotypes by internal standardization and dilution analysis according to Jones (1987). The results (data not shown) indicated the absence of potentially interfering compounds in the aqueous leaf extracts of the three genotypes (Prof. W. Davies, personal communication).
Electrolyte leakage measurements
Fifteen leaf discs (0.8 cm²) per sample were cut from well watered plants, rinsed three times with deionized water, and floated for 17 h on deionized water (control), or on a 30% (w/v) PEG MW 8000 solution (osmotic potential: -1.54 MPa). After washing, the discs were allowed to float on deionized water for 24 h. During this period leakage was monitored with a conductimeter (Crison 522, Crison Instruments, Spain). Injury index (I%) was calculated according to the formula I%=[1-(T-D/T-W)]x100, where D and W represent the electrolytes released by PEG-dehydrated and watered (control) samples, respectively, and T the total electrolyte content measured after heating the control samples at a temperature of 90ºC for 2 h, followed by cooling until ca. 20ºC, as described in Campos and Pham Thi (1997).

Lipid analysis
Leaf samples (2 g FW) were boiled 2 min in deionized water, in order to stop lipolytic activities. Lipids were extracted in chloroform/methanol /water (1/1/1, by vol.) according to Allen et al. (1966). After evaporation of chloroform under nitrogen, the lipophilic extracts were resuspended in ethanol:toluene (1/4, by vol.) and stored at -20ºC, for further analysis. Fatty acids were saponified, methylated with BF₃ (Merck) using heptadecanoic acid (C17:0) as an internal standard (Metcalfe et al., 1966), and analysed with a gas chromatograph (Unicam 610, Unicam Ltd., U.K.), equipped with a flame-ionization detector, as described in Campos et al. (2003). The fatty acid methyl esters were separated on a DB-Wax column, (J&W Scientific, U.S.A., 0.25 mm i.d. x 30 m, 0.25 μm) with programmed temperature. Carrier gas was hydrogen with a flow rate of 1 ml min⁻¹, at a split ratio of 1:100 of the sample.

For lipid class analysis, a mixture of three samples was prepared for each treatment and analysed in triplicate. Separation was performed by thin layer chromatography on G60 silicagel plates (Merck) in chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5, by vol.) according to Lepage (1967), and subsequently in petroleum ether/diethyl ether/acetic acid (70/30/0.4, by vol.), according to Mangold (1961), to obtain a clear separation between MGDG and neutral lipids. After spraying the plates with primuline 0.01% in 80% acetone and visualisation under UV, the bands were scraped off, saponified, methylated and analysed by gas liquid chromatography, as described above.

Statistical analysis
The data were analysed statistically using a two-way ANOVA, applied to the various measured and calculated parameters, followed by a Tukey test for mean comparison between genotypes or degrees of dehydration (for a 95% confidence level).

Results
Stomatal conductance
Well-watered plants of the three genotypes presented similar gₛ values (ca. 360-370 mmol H₂O m⁻² s⁻¹), but their responses varied along the dehydration period (Figure 1). At early stages of water deficit (ca. 85% RWC), cvs. EPACE-1 and 1183 presented lower gₛ values than Vg. Such difference of behavior was maintained for RWC values around 80%, when gₛ remained high in Vg (ca. 300 mmol H₂O m⁻² s⁻¹) while EPACE-1 and 1183 presented already gₛ values below 100 mmol H₂O m⁻² s⁻¹. In Vg similar lowered gₛ values were observed only for RWC ca. 72-75%.

Leaf ABA content
Under control conditions, EPACE-1 and 1183 presented a somewhat higher ABA content than Vg, which remained low until 80% RWC (Figure 2). Furthermore, for higher dehydration levels Vg displayed a stable value of around 3 µg g⁻¹ DW, whereas a contrasting response was observed for EPACE-1 and 1183. In fact, in these cvs. ABA content started to rise from the very early stages of dehydration (RWC between 90-80%). Maximal values (ca. 4-6 µg g⁻¹ DW) were reached under moderate water deficits in EPACE-1, being maintained with severe drought. For cv. 1183 a continuous ABA accumulation occurred with the imposition of stronger water deficits, reaching ca. 11.5 µg g⁻¹ DW under severe stress conditions (ca. 6 fold increase in relation to control).
Figure 1. Relation between decreasing leaf relative water content (RWC) and stomatal conductance ($g_s$) in *V. glabrescens* (Vg) and *V. unguiculata* (cvs. 1183 and EPACE-1). Each value represents an individual measurement and lines correspond to the best fit regression.

**Electrolyte leakage test**

The three genotypes were affected differently by PEG-induced dehydration (Figure 3) from the beginning of measurements. After the 3rd hour, significant leakage differences were already observed when comparing cv. 1183 with Vg and EPACE-1. By the 5th hour I% values of 41%, 24%, and 19% were obtained respectively for 1183, EPACE-1 and Vg. Differences in I% values were further amplified after 24 h, reflecting a much stronger impact of dehydration on membrane structure and integrity in cv. 1183.
Figure 2. Changes in leaf endogenous abscisic acid (ABA) content in *V. glabrescens* (Vg) and *V. unguiculata* (cvs. 1183 and EPACE-1) with decreasing relative water content (RWC). Each value represents an individual measurement.
Lipid analysis

Total fatty acid (TFA) content was similar in control plants of the three genotypes (Fig. 4). Under mild stress (S1, RWC = 65-75%), TFA sharply increased (45%) in Vg, due to increase in all polar lipid contents, particularly in phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylcholine (PC), that showed increases of 167%, 96% and 82%, respectively, when compared to control plants (Figure 5). As regards EPACE-1, although TFA content remained unaltered under mild stress (Figure 4), a 27% decrease in MGDG occurred that was counterbalanced by an increase of the two main phospholipids PC (50%) and PG (42%), as well as in PI (43%). In the case of 1183, a gradual decrease of TFA was observed in parallel to the increase of drought severity. Under S1 conditions, drastic reductions were found for MGDG (56%), PC (36%) and PG (46%), that were only partially compensated by the increases in DGDG (6%), PE (128%) and PI (128%), therefore resulting in a TFA reduction of 20%.
At severe water deficit (S2, RWC < 60%), a significant 24% TFA reduction was observed in Vg in relation to its control values (although that decrease reached 48% when compared to S1), whereas EPACE-1 showed decreases of 20% and 17%, when compared to control and S1, respectively (Figure 4). Cv. 1183 presented a gradual TFA decrease, reaching a 52% and 39% lower values in comparison to control and S1 stages, respectively. Under S2 conditions, the observed impact was linked to decreases in all lipid classes, except in EPACE-1 (Figure 5), where only galactolipids (mostly MGDG) content decreased. It should be noticed that MGDG was the most drought-susceptible lipid throughout the whole drought period and in all genotypes, showing reductions higher than 50% at S2 (Figure 5).

Figure 5. Changes in lipid classes content of V. glabrescens (Vg) and V. unguiculata (cvs. EPACE-1 and 1183) leaves in response to three water regimes: C: RWC > 90%; S1: RWC 75-65%; S2: RWC < 60%. Values are the mean ± SE (n=9). MGDG, monogalactosyl-diacylglycerol; DGDG, digalactosyl-diacylglycerol, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PG, phosphatidylglycerol, PI, phosphatidylinositol.
Table 1. Effects of water stress on the fatty acid composition (mol %) of galactolipid classes monogalactosyl-
diacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG) in V. unguiculata (cvs. 1183 and EPACE-1) and V.
glabrescens (Vg) leaves. Mean values (n = 3) followed by different letters express significant differences between
dehydration levels within a genotype (a, b, c) or between genotypes with the same dehydration degree (r, s, t), based
upon Tukey’s HSD means separation test at P < 0.05; a and r represent the highest values. C: RWC > 90%; S1: RWC 75-
65%; S2: RWC< 60%.

| Genotype | Lipid | Treatment | C16:0 | C16:1c | C18:0 | C18:1 | C18:2 | C18:3 |
|----------|------|----------|-------|--------|-------|-------|-------|-------|
| Vg       | MGDG | C        | 3.03  | 0.43   | 0.21  | 0.25  | 3.55  | 92.53 |
|          | S1   | 2.96     | 0.36  | 0.86   | 0.45  | 0.20  | 6.09  | 89.27 |
|          | S2   | 3.38     | 0.40  | 0.66   | 0.20  | 0.62  | 3.45  | 89.09 |
|          | DGDG | C        | 30.48 | 4.19   | 2.20  | 0.74  | 7.95  | 54.45 |
|          | S1   | 21.82    | 1.71  | 3.24   | 1.10  | 1.63  | 6.53  | 63.51 |
|          | S2   | 24.41    | 3.71  | 2.88   | 1.00  | 7.43  | 60.57 |
| EPACE-1  | MGDG | C        | 1.95  | 0.37   | 0.10  | 0.37  | 1.41  | 95.81 |
|          | S1   | 2.36     | 0.13  | 0.48   | 0.15  | 3.17  | 93.71 |
|          | S2   | 3.37     | 0.40  | 0.53   | 0.19  | 4.24  | 91.27 |
| 1183     | MGDG | C        | 2.65  | 0.30   | 0.23  | 0.15  | 1.66  | 95.00 |
|          | S1   | 2.86     | 0.87  | 0.49   | 0.33  | 2.31  | 93.14 |
|          | S2   | 2.76     | 3.67  | 0.46   | 0.42  | 3.19  | 85.30 |
|          | DGDG | C        | 20.02 | 4.61   | 0.97  | 0.17  | 1.01  | 73.22 |
|          | S1   | 30.67    | 1.22  | 2.52   | 0.80  | 6.21  | 58.57 |
|          | S2   | 31.58    | 1.34  | 1.97   | 1.18  | 8.29  | 55.62 |

In parallel to the above presented class content modifications, the weight of the main FA within
galactolipid classes also changed with drought implementation. In control plants, linolenic acid
(C18:3) percentage in MGDG molecules did not differ between the three genotypes, but was
significantly reduced by 10%, 5% and 4% for 1183, EPACE-1 and Vg, respectively, under severe
drought (Table 1). As regards the C18:3 percentage in DGDG, Vg presented the lowest values in
control plants (Table 1), which increased under S1 and S2 conditions. In 1183 the percentage gradually
decreased under moderate and severe stress, while in EPACE-1 a reduction occurred only under S2.
Such decreases occurred concomitantly to increases in the percentage of more saturated fatty acids,
namely linoleic acid (C18:2) and palmitic acid (C16:0).

Concerning phospholipid classes, no noticeable change was found in their FA acid composition, except a decrease in the percentage of 16:1r in PG (data not shown).

Discussion

For the studied cvs., there appear to be two mechanisms in Vigna sp. to cope with drought in
what concerns stomatal control to water loss. In the first, plants close their stomata at the early stages of
dehydration, as was the case of V. unguiculata cvs. (1183 and EPACE-1). In the second plants tolerate
a lower water availability without strong g, reduction, as happened in V. glabrescens. Such stomatal closure at moderate dehydration (80%
RWC), occurring in V. unguiculata cvs. 1183 and EPACE-1, was probably controlled by endogenous leaf ABA that increased in both genotypes.

Electrolyte leakage test showed that membrane integrity under PEG-induced dehydration was better preserved in V. glabrescens and V.
unguiculata EPACE-1, suggesting that both genotypes present a higher protoplasmic tolerance to dehydration than cv. 1183.

Drought impact on membrane lipids further points to a higher sensitivity of cv. 1183 due to a large reduction of TFA. On the other hand, under
S1, total lipid content of leaves remained unchanged in EPACE-1 and increased in Vg, being
affected only in S2 conditions (and to a lower extent than cv. 1183). In Vg de novo lipid biosynthesis under S1 reflected a general rise in all lipid classes, particularly in DGDG. Such higher DGDG content may represent an important adaptive mechanism. Effectively, DGDG is a bilayer forming lipid involved in membrane stabilization, regulation of ionic permeability and preserving activity of membrane proteins (Webb and Green, 1991; Siegenthaler and Trémolières, 1998). Also, a reduction of the MGDG to DGDG
ratio, linked to DGDG synthesis, was reported in drought tolerant genotypes under mild water deficit (Torres-Franklin et al., 2007) and under cold (Harwood, 1998). Furthermore, the newly synthesized DGDG in Vg showed qualitative modifications, as it presented a raise of the highly unsaturated C18:3. This may contribute for the lower photosynthetic impairments previously observed in this genotype under drought (Campos et al., 1999a). It is generally accepted that preponderance of unsaturated fatty acids enhances fluid phase in membranes, maintaining biological activity under stressfull conditions (Leshem, 1992; Murata and Wada, 1995; Somerville, 1995; Partelli et al., 2011), as photosynthetic performance is tightly associated to membrane lipid composition (Horváth et al., 1987). Water stress favours membrane rigidity (Webb and Green, 1991; Leone et al., 1996), and a compensatory increase in acyl unsaturation may contribute to maintain lipid acyl motion in thylakoid membranes, therefore preserving the rate of plastoquinone diffusion and ensuring photosynthetic electron transport (Harwood, 1998; Siegenthaler and Trémolières, 1998).

V. unguiculata cv. 1183 was more susceptible to PEG-induced dehydration than the other genotypes, suggesting a loss of selectivity. That could have been related to TFA decrease which in turn was a result of PC, PG and, mostly MGDG decreases in S1 conditions, that was extended to other lipid classes in S2. Furthermore, linolenic acid (C18:3) weight in MGDG (and DGDG) decreased under severe drought. That suggested an inhibition of lipid biosynthesis and/or an acceleration of degradative phenomena particularly as regards chloroplast membranes, probably linked to oxidative and hydrolytic reactions already described in Vigna species (Monteiro de Paula et al., 1993; Ferrari-Iliou et al., 1994; Sahsah et al., 1998; Maarouf et al., 1999). Such a low capacity to withstand MGDG degradation agrees with our previous report on the presence of higher galactolipase activities in water stressed plants of 1183 in comparison with EPACE-1 (Sahsah et al., 1998; Matos et al., 2001) and Vg (Campos et al., 1999b). Therefore, the results point that in cv. 1183 membrane repair and/or protection mechanisms might be impaired or insufficient to cope with increasing dehydration, leading to poor membrane preservation, which in turn compromises cell compartmentation and functioning.

Drought induced increasingly higher ABA contents in 1183, which may have contributed to accelerate senescence, resulting in cell damage. A similar mechanism was suggested to occur in the drought-susceptible Mediterranean shrub Lavandula stoechas L. (Lopez-Carbonell et al., 1996). ABA effects on cell metabolism are multiple and sometimes contradictory (Wilkinson and Davies, 2002). Apart from eliciting stomatal closure and triggering cell signalling pathways (Himmelbach et al., 2003) which stimulate beneficial responses, ABA can also cause deleterious effects in plant development, due to its involvement in leaf senescence and abscission (Trewawas and Jones, 1991). Jiang and Zhang (2002) remarked that treatment of maize seedlings with low concentrations of ABA induced antioxidative defence response, whereas high ABA concentrations led to excessive generation of activated oxygen species.

More dehydration-tolerant membrane systems in Vg and EPACE-1 seem to be related to stable and lower ABA contents under increasing water deficit (RWC < 80%). The question raises if, in these tolerant genotypes, ABA could be involved in the preservation of membrane integrity, therefore contributing to enhance membrane tolerance. Our previous experiments carried out in vitro using Vigna leaf discs highlighted a protective effect of exogenously applied ABA against membrane lipid degradation under dehydration conditions (Campos and Pham Thi, 1997). Direct effects of ABA on artificial membrane systems have been reported, leading for example to increases in microviscosity (Shripathi et al., 1997) and permeability (Schauf et al., 1987). Membrane stability may be achieved through binding of ABA molecules to specific lipid components (Wassal et al., 1985; Leshem, 1992), or through ABA involvement in lipid metabolism (Aghofack-Nguemezi et al., 1991; Zou et al., 1995), although the mechanisms by which ABA act are still unknown. Some evidence has been reported concerning the effects of ABA on the regulation of genes coding for enzymes of lipid metabolism (Matos et al., 2001; Matsuda et al., 2001; Wang, 2002; Narusaka et al., 2003). Studies using cDNAs microarrays showed that ABA triggers expression of a large set of genes (Seki et al., 2002; Rabbani et al., 2003). Several of these genes are linked to lipid degradation, through generation of lipid second messengers by phospholipases (Sanchez and Chua, 2001; Wang, 2002). Long-term modifications of lipid metabolism may also result from these ABA-induced phenomena Aghofack-Nguemezi et al., 1991; Zou et al., 1995). A possible involvement of ABA in the induction of antioxidant enzymes

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should not be excluded (Jiang and Zang, 2002), and could also result in lipids preservation.

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