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The stay green mutations *d1* and *d2* increase water stress susceptibility in soybeans

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Abstract

The stay green mutant genotype *d1d1d2d2* inhibits the breakdown of chloroplast components in senescing leaves of soybean (*Glycine max* L. Merr.). Together with *G* (a gene that preserves chlorophyll in the seed coat) they may extend photosynthetic activity in some conditions. While wild-type soybeans maintain high leaf water potentials right up to abscission, leaves of *(GG)d1d1d2d2* dehydrate late in senescence, which suggests that water relations may be altered in the mutant. Three-week-old plants were subjected to a moderate water deficit (soil water potential = −0.7 MPa) for 7–10 d. Leaf water potential and relative water content decreased significantly more in response to water deficit in unifoliate leaves of *GGd1d1d2d2* than in a near-isogenic wild-type line. Down-regulation of stomatal conductance in response to drought was similar in mutant and wild-type leaves. Likewise, exogenously applied ABA reduced stomatal conductance to a similar extent in the mutant and the wild type, and applied ABA failed to restore water deficit tolerance in *GGd1d1d2d2*. Experiments with explants lacking roots indicate that the accelerated dehydration of *GGd1d1d2d2* is probably not due to alterations in the roots. In a comparison of near-isogenic lines carrying different combinations of *d1*, *d2 and G*, only *d1d1d2d2* and *GGd1d1d2d2* (i.e. the genotypes that cause the stay green phenotype) were more susceptible to water deficit than the wild type. These data suggest that pathways involved in chloroplast disassembly and in the regulation of stress responses may be intertwined and controlled by the same factors.

Key words: Drought, senescence, soybean, stay green, stress tolerance.

Introduction

Cellular components are broken down during senescence, starting with chloroplasts which are the first organelles to show clear signs of deterioration. Senescing chloroplasts undergo an orderly and co-ordinated decline in the levels of photosynthetic proteins, pigments and lipids, and chloroplasts eventually disintegrate in the final stages of senescence (Noodén *et al*., 1997). Chloroplast disassembly has received considerable attention because of its negative impact on photosynthesis and its role in nutrient redistribution within the plant (Noodén *et al*., 1997; Thomas and Howarth, 2000).

Chloroplast degradation is under genetic control, as shown by the natural occurrence of mutations that interfere with the degradation of chloroplast components in various species (Thomas and Smart, 1993; Noodén and Guiamét, 1996; Thomas and Howarth, 2000). In soybean, the homozygous combination of recessive mutations at the *d1* and *d2* loci (i.e. the *d1d1d2d2* genotype) inhibits the degradation of chlorophyll, chlorophyll-binding proteins and Rubisco (Guiamét *et al*., 1991; Guiamét and Giannibelli, 1994, 1996). The addition of the dominant *G* mutation at a third locus (i.e. the *GGd1d1d2d2* genotype) retards the senescence-associated decline of photosynthetic activity in growth-chamber experiments (Guiamét *et al*., 1990). The *d1d1d2d2* and *GGd1d1d2d2* stay green genotypes (abbreviated *d1d2* and *Gd1d2*, respectively) inhibit chloroplast degradation during normal monocarpic senescence and also in detached leaves induced to senesce by prolonged incubation in darkness (Guiamét and Gianibelli, 1994). Interestingly, while they inhibit the degradation of a wide range of chloroplast components, *d1d2* and *Gd1d2* apparently have no effect extending the life span of leaves, i.e. the timing of leaflet abscission is not affected by these mutations (Guiamét *et al*., 1990).

There are only a few studies on the changes in the water relations of leaves or plants during senescence (Zur

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et al., 1981; Neumann and Stein, 1984; Neumann, 1987; Thomas *et al*., 1991). It is well known that stomatal conductance declines during senescence (Gepstein, 1988), although it is not clear to what extent this represents only a downward adjustment to reduced photosynthetic capacity (Thomas *et al*., 1991). In the absence of water deficit, leaf water potential changes very little, or not at all, during senescence in species where the life span of leaves is terminated by abscission, as in soybeans and other dicots (Guiamét *et al*., 1990). Even if water potential does not change, solute potential and hydraulic conductivity of the xylem decrease during senescence in some legumes (Zur *et al*., 1981; Neumann and Stein, 1984; Neumann, 1987). In most monocots and in dicots where leaves do not abscise, leaves normally dehydrate late during senescence.

While the water potential of wild-type soybean leaves remains constant throughout senescence, leaves of the stay greens *d1d2* and *Gd1d2* dehydrate very late in senescence, i.e. a few days before leaf shedding (Guiamét *et al*., 1990). Furthermore, plants of *Gd1d2* growing outdoors under ambient conditions of irradiance, temperature and relative humidity, normally exhibit reduced stomatal conductance and transpiration rates compared to near-isogenic wild-type plants of the same age (Luquez and Guiamét, 2001). This suggests that *Gd1d2* might have pleiotropic effects interfering with the regulation of water balance.

In this paper the responses of *Gd1d2* to moderate soil water deficits were examined and it was found that there are pleiotropic effects of *d1* and *d2* that reduce water stress tolerance in the stay green mutant. The results suggest that the *d1* and *d2* mutations represent genetic lesions in a pathway controlling chloroplast disassembly and leaf water balance.

Materials and methods

Plant material and growth conditions

Soybean seeds of wild type cv. Clark (genotype *ggD1D1D2D2*) and near-isogenic lines carrying different combinations of the stay green genes *G*, *d1* and *d2* were obtained from the Soybean Germplasm Collection, Department of Agronomy, University of Illinois, Urbana, IL, USA (Bernard *et al*., 1991). The near-isogenic lines used in this study were developed by Dr RL Bernard using cv. Columbia as the donor of the *G*, *d1* and *d2* mutations backcrossed six times to cv. Clark as the recurrent parent. Seeds were planted in pots with soil and allowed to germinate and develop in a greenhouse for 1 (vegetative plants) or 7 (reproductive plants) weeks. Then they were transferred to a growth chamber (300–400 μmol m^{-2} s^{-1} of photosynthetically active radiation, 26/20 °C day/night temperature, 10 h photoperiod) for the duration of the experiments. Seeds were not inoculated prior to planting, but pots were regularly fertilized with an N–P–K (15–15–15) fertilizer. In some experiments, soybean explants consisting of a piece of stem with one node and subtending leaf and pods (Neumann *et al*., 1983) were taken from plants at mid–late pod fill and cultured in distilled water under the same conditions as described above. The end of the stem was re-cut under water every 2 d to avoid callus or root formation.

Water stress treatment

Water stress was imposed by withholding watering until soil water potential reached −0.7 MPa. Thereafter, soil water potential was maintained around −0.7 MPa by weighing pots to estimate and replace the amount of water lost every day. Soil water potential was measured with a Wescor HR 33T Dew Point Hygrometer and PST-55 soil probes.

Leaf water status and transpiration

Leaf water potential was measured with a Wescor HR 33T Dew Point Hygrometer and C-52 leaf chambers. To estimate the extent of osmotic adjustment, leaves were detached and their petioles dipped in distilled water for 4 h to reach maximum turgor. Leaves were then wrapped in aluminium foil, frozen at −20 °C for 1 h, and allowed to thaw at room temperature. The cell sap was extracted by pressing the leaf in a syringe barrel fitted with glass wool at the outlet to filter out cell debris. Sap was collected in 5 mm diameter discs of filter paper, the discs were placed in C-52 chambers and cell sap water potential was measured with a Wescor HR 33T Dew Point Hygrometer. Relative water content was calculated (Luquez *et al*., 1997). Stomatal conductance and transpiration rate were measured with a Li-Cor LI 1600 steady state porometer.

ABA treatment

ABA was supplied to plants subjected to water stress in hydroponic culture. Non-inoculated seeds were germinated on filter paper for 4 d and then transferred to a hydroponic culture system (Leggett and Frere, 1971). Water stress was imposed on 3-week-old plants by adding polyethylene glycol 4000 to the nutrient solution in steps to reach −0.1, −0.3 and −0.5 MPa after 1, 3 and 6 d, respectively. Abscisic acid (10^{-6} M) was added to the nutrient solution in half of the pots 24 h before the start of the water stress treatment.

Western blotting

Leaves were ground in buffer (TRIS 50 mM pH 7.5, EDTA 1 mM, PVPP 1% w/v, β-mercaptoethanol 0.1% v/v, phenylmethylsulphonylfluoride 1 mM, and leupeptin 0.1 mM), the homogenate was centrifuged at 10 000 *g* for 10 min and the supernatant was mixed with an equal volume of denaturing buffer (TRIS 125 mM pH 6.8, SDS 4% w/v, β-mercaptoethanol 10% v/v, glycerol 10% v/v) and boiled for 2 min. Proteins were separated in 13% acrylamide minigels, transferred to nitrocellulose membranes and probed with an anti-dehydrin antibody (Close *et al*., 1993). Blots were developed with a chemiluminescence detection kit as described previously (Tambussi *et al*., 2000).

Results

Water stress susceptibility in Gd1d2

Initially, plants were subjected to a treatment of moderate soil water deficit (−0.7 MPa) at the beginning of pod

Table 1. *Percentage of leaflets with 50% of the area visibly dry in the first trifoliate leaf of wild-type cv. Clark and* Gd1d2 *soybeans subjected to water stress (soil water potential = −0.7 MPa) for 10 d*

The water stress treatment started at mid-pod fill (54 d after planting). Means followed by the same letter do not differ significantly at 5% (LSD test).

	Percentage of leaflets more than 50% dry Day 10
Clark control	3.5 a
Clark water-stressed	5.4 a
Gd1d2 control	1.8 a
Gd1d2 water-stressed	26.0 b

filling (54 d after planting). At the end of a 10 d drought period a relatively large percentage of leaflets had more than 50% of their area visibly dry in water-stressed plants of *Gd1d2*, while the percentage of dry leaflets was much smaller in wild type cv. Clark (Table 1). Thus, substantial leaf dehydration took place in *Gd1d2* at a level of water deficit that produced virtually no visible dehydration in the wild type. Similar experiments were carried out to test susceptibility to water deficit in 3-week-old plants. Pre-dawn water potential and relative water content of unifoliate leaves started to decrease 3 d and 4 d after withholding watering, respectively (Fig. 1). Seven days after the start of the water deficit treatment, leaf water potential decreased from −1.1 to −2.2 MPa, and relative water content from 95% to 72%, in unifoliate leaves of the wild type. Over the same period, the water potential dropped to −3.2 MPa and the relative water content to 51% in *Gd1d2* (Fig. 1).

The faster decline of leaf water content in *Gd1d2* was not due simply to differences in the rate of soil water consumption. For example, 6 d after the start of the stress treatment the soil reached a water potential of −0.7 MPa in pots of the mutant and the wild type, yet the leaf water potential and the relative water content were significantly lower in *Gd1d2* (Fig. 1). Thus, *Gd1d2* was more susceptible to a moderate water deficit than its near-isogenic wild-type line cv. Clark.

Stomatal conductance and osmotic adjustment in Gd1d2

Abnormal regulation of stomatal closure in response to water deficit might cause the accelerated dehydration of *Gd1d2*. Therefore, changes in stomatal conductance (g_s) and transpiration rate (E) were measured in plants subjected to water deficit. Midday transpiration rates and stomatal conductance declined in unifoliate leaves of well-watered soybean plants between weeks 3 and 4 after germination (Fig. 2) probably reflecting an ontogenic shift in stomatal conductance. In plants subjected to water deficit, g_s and E declined significantly in the first 3 d

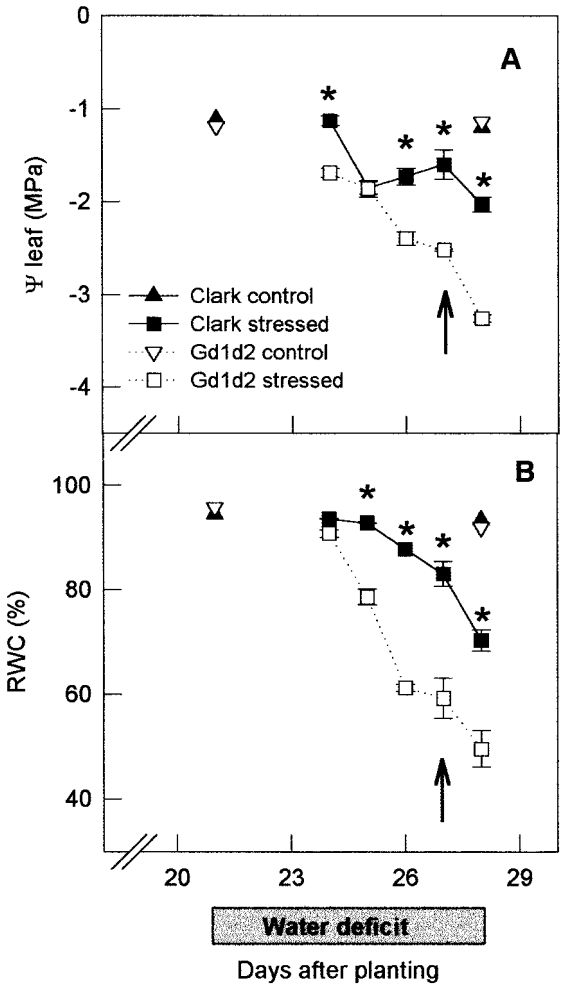

Fig. 1. Water potential (Ψ_{leaf}, A) and relative water content (*RWC*, B) in unifoliate leaves of wild-type cv. Clark and *Gd1d2* soybeans subjected to water deficit. Vertical bars indicate the standard error of the mean. Arrows mark the time when soil water potential reached −0.7 MPa. Asterisks indicate significant differences at 5% level (LSD test) between water-stressed leaves of wild-type cv. Clark and *Gd1d2*.

after withholding watering, and they remained significantly lower than in control plants thereafter. There were no significant differences between wild type cv. Clark and *Gd1d2* in midday g_s or E of water-stressed leaves. Similar results were obtained with plants subjected to water deficit during their reproductive period (data not shown). Thus, increased susceptibility to water deficit in *Gd1d2* occurs in spite of normal down-regulation of stomatal aperture to adjust water consumption to reduced soil water supply.

Plant tissues accumulate solutes (i.e. osmotic adjustment) to reduce water potential and maintain growth and water absorption from increasingly dry soils (Munns and Sharp, 1993; Mullet and Whitsitt, 1996; Nilsen and Orcutt, 1996). To test if *Gd1d2* differed from the wild type in its capacity for osmotic adjustment, the solute potential of leaves was measured in control and stressed leaves of both genotypes. Prior to the measurements, the leaves

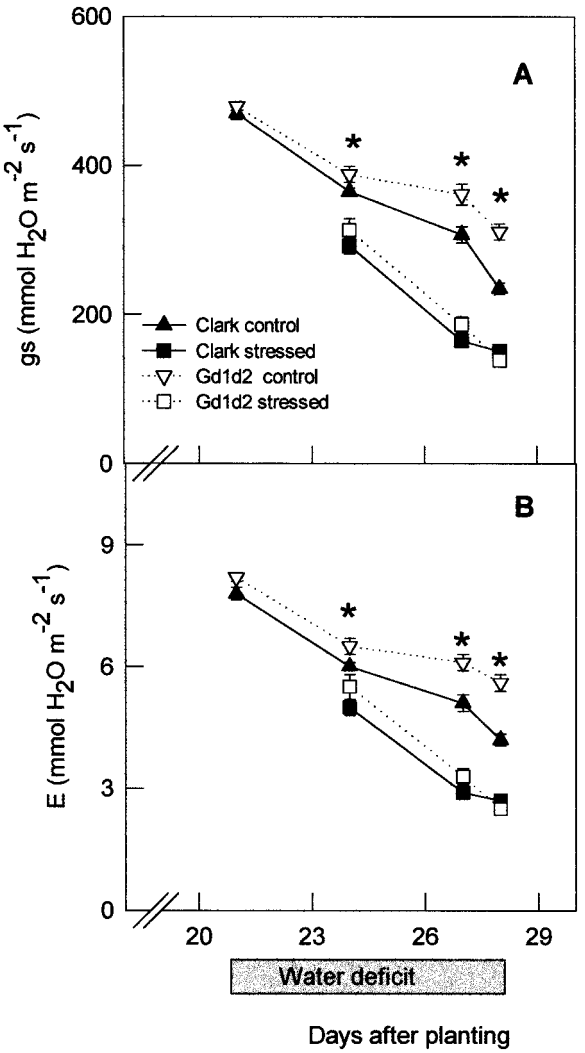

Fig. 2. Stomatal conductance (g_s, A) and transpiration rate (E, B) in unifoliate leaves of wild-type cv. Clark and *Gd1d2* soybeans subjected to water deficit. Vertical bars show the standard error of the mean. There were no statistically significant differences between genotypes in either treatment, therefore, asterisks indicate significant differences at 5% level (LSD test) between control and water-stressed leaves.

were allowed to recover full turgor in order to distinguish the true accumulation of solutes from solute concentration due simply to water loss and decreased cell volume. Table 2 shows that there were no significant changes in solute accumulation in response to water deficit, or between the wild type and stay green mutant, implying that mature soybean leaves did not undergo osmotic adjustment in response to water deficit.

Leaf dehydration in explants

Alterations in the roots or stem causing reduced water supply to the leaves might be involved in the higher susceptibility to water deficit of the stay green mutant. Soybean explants consisting of a piece of stem and subtending leaf might allow the examination of water

Table 2. *Osmotic adjustment in leaves of wild-type cv. Clark and* Gd1d2 *soybeans subjected to water deficit*

Leaf water (Ψ_{leaf}) and solute potential (Ψ_{solute}) were measured at midnight in unifoliate leaves during an episode of moderate water stress. To distinguish true accumulation of solutes from solute concentration due simply to water loss and decreased cell volume, leaves were rehydrated for 4 h to reach maximum turgor before cell sap extraction. For each date, means followed by the same letter do not differ significantly at 5% (LSD test).

	Day 0		Day 7		Day 9	
	Ψ_{leaf}	Ψ_{solute}	Ψ_{leaf}	Ψ_{solute}	Ψ_{leaf}	Ψ_{solute}
Clark control	−1.0 a	−1.1 a	−0.8 a	−1.3 a	−0.9 a	−1.5 a
Clark water-stressed			−1.7 b	−1.6 a	−1.7 b	−1.5 a
Gd1d2 control	−1.0 a	−1.3 a	−0.7 a	−1.4 a	−1.0 a	−1.5 a
Gd1d2 water-stressed			−2.0 b	−1.5 a	−1.9 b	−1.4 a

stress susceptibility without the possible interfering effects of the roots. However, attempts to impose water stress on these explants by adding polyethyleneglycol (PEG) 4000 to the medium resulted in an almost immediate dehydration of all explants, mutant and wild type alike (data not shown), probably because PEG 4000 taken up through the cut end of the stem clogged the xylem. However, leaf dehydration is also manifested prior to absicission in leaves of well-watered plants of *Gd1d2* (Guiamét *et al.*, 1990). The same physiological and molecular alterations probably underlie the increased susceptibility to water deficit and leaf dehydration before abscission. Therefore, podded explants (Neumann *et al.*, 1983) were excised at late pod fill (66 d after planting) and used to test if the roots impose limitations to water flow that affect adversely the water balance in *Gd1d2*. Although explant leaves in water senesced faster than comparable leaves of intact plants (Neumann *et al.*, 1983), their behaviour in terms of leaf dehydration was quite similar. Leaves dehydrated prior to abscission in *Gd1d2* (Fig. 3) whether the leaves were attached to intact plants (i.e. with roots) or to explants (without roots), in contrast to cv. Clark where leaves were shed fresh. Thus, alterations of the roots do not seem to play a significant role in the dehydration of *Gd1d2* leaves before abscission and, likewise, the roots are probably not involved in the increased susceptibility to water deficit of the mutant.

Responses of Gd1d2 *to exogenously applied abscisic acid*

Abscisic acid (ABA) participates in many adaptive responses to water stress, including stomatal closure and the synthesis of dehydration-induced proteins (Vartanian, 1996), and it may also promote leaf senescence in some species (Noodén, 1988). A deficiency in abscisic acid or the inability to respond to ABA might account for the stay green trait and water deficit susceptibility of *Gd1d2*. Plants of the stay green and wild type were subjected to

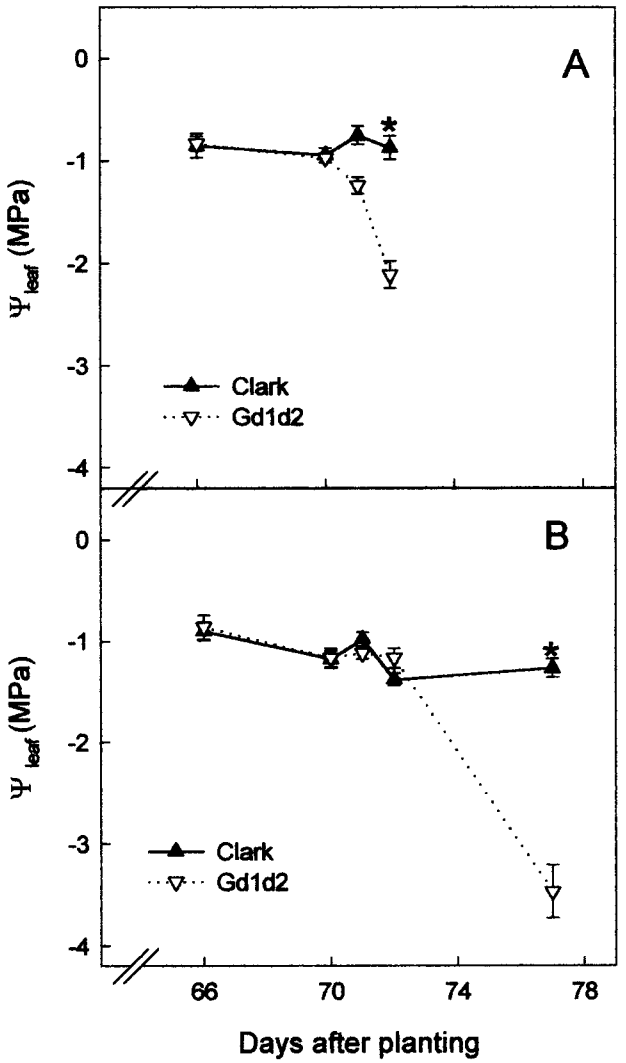

Fig. 3. Leaf water potential (Ψ_{leaf}) of soybean explants (A) taken from plants of wild-type cv. Clark and *Gd1d2* at late pod fill (66 d after planting). Explants consisted of the third trifoliate leaf (counting from the base), a piece of internode below the leaf and subtending pods. (B) The water potential of the same leaves attached to intact plants. Vertical bars show the standard error of the mean. Asterisks indicate significant differences at 5% level (LSD test).

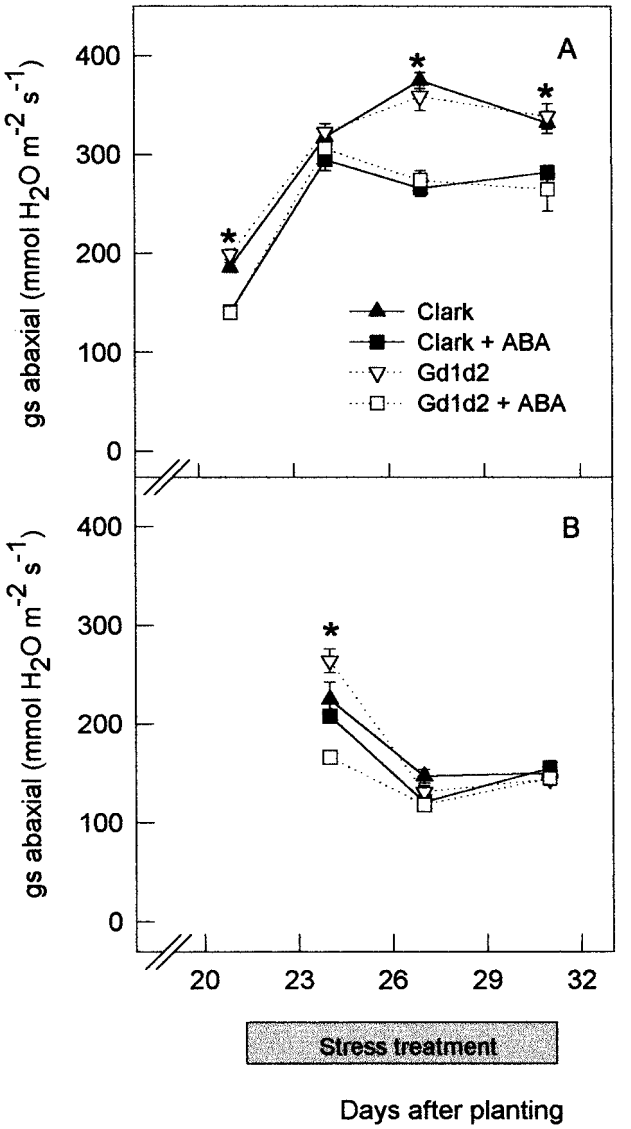

Fig. 4. Stomatal conductance (g_s) of the abaxial surface of unifoliate leaves of wild-type cv. Clark and *Gd1d2* plants subjected to water stress in hydroponic culture. (A) Control plants in nutrient solution and (B) hydroponically cultured plants stressed with PEG (water potential: −0.5 MPa). Some pots were supplied with ABA (10^{-6} M). Vertical bars show the standard error of the mean. In (A) asterisks indicate significant differences at 5% level (LSD test) between leaves treated with or without ABA, irrespective of the genotype. In water-stressed plants (B) ABA caused a statistically significant decrease in g_s only for *Gd1d2* leaves on day 24 (shown by the asterisk).

water deficit in a hydroponic system that allowed the nutrient solution to be supplemented with ABA. While g_s tended to decrease with age in 3-week-old plants growing in soil, for unknown reasons g_s tended to increase between days 21 and 24 in plants cultured in a hydroponic system (Fig. 4). The addition of ABA (10^{-6} M) reduced stomatal conductance of non-stressed leaves in both genotypes (Fig. 4). In water-stressed plants, ABA caused a modest but significant decrease of g_s in *Gd1d2* 3 d after the start of the treatment, but thereafter g_s continued to decrease in non-treated plants and ABA did not cause any additional decrease of g_s, suggesting that the response was saturated by endogenous ABA produced in response to water deficit (Dodd *et al.*, 1996). As in plants undergoing water stress in soil, relative water content decreased more in leaves of *Gd1d2* than in the wild-type cv. Clark (Table 3), and the faster dehydration of *Gd1d2* was not prevented by ABA. While partial closure of stomata in non-stressed plants supplied with ABA indicates that *Gd1d2* responds to ABA, the inability of exogenous ABA to protect *Gd1d2* leaves against water deficit suggests that endogenous levels of ABA may be normal in *Gd1d2* and that water deficit susceptibility in the stay green mutant is not related to alterations in ABA metabolism or response.

Accumulation of dehydrins in response to water deficit

Water stress induces the accumulation of several dehydration-related proteins, including dehydrins, some of which might be involved in maintaining cell integrity (Bartels *et al.*, 1996). Three dehydrins of apparent molecular masses of 34, 30 and 27 kDa were strongly induced by water stress in soybean, and levels of these drought-induced dehydrins were even higher in stressed leaves of *Gd1d2*, than in the wild-type cv. Clark (Fig. 5), which is consistent with the lower water potential of mutant leaves subjected to water deficit. Similar results were obtained with plants subjected to water deficit at mid pod filling (data not shown).

Table 3. *Relative water content in unifoliate leaves of 3-week-old plants of wild-type cv. Clark and* Gd1d2 *cultured hydroponically, supplied or not with ABA (10^{-6} M), and subjected to water stress through application of PEG 4000 (water potential: −0.5 MPa)*

DAP: days after planting. For each date, values followed by the same letter do not differ significantly at 5% (LSD test).

		Relative water content (%)		
	ABA treatment	Day 0 (21 DAP)	Day 6 (27 DAP)	Day 9 (30 DAP)
Clark control	No	96.1 a	96.2 a	96.6 a
	Yes	96.6 a	96.0 a	96.8 a
Clark stressed	No		93.0 a	91.0 b
	Yes		93.0 a	89.6 b
Gd1d2 control	No	94.9 a	94.8 a	95.3 a
	Yes	96.6 a	95.5 a	96.4 a
Gd1d2 stressed	No		88.2 ab	81.2 c
	Yes		83.0 b	80.2 c

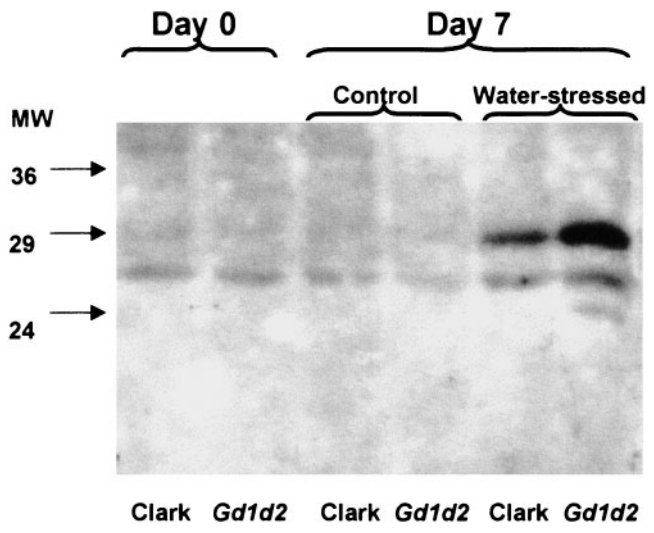

Fig. 5. Western blot of soluble proteins of unifoliate leaves of wild-type cv. Clark and *Gd1d2* soybeans probed with an anti-dehydrin antibody. Samples were taken at the start of the water deficit treatment (day 0) and after 7 d. MW: molecular mass markers.

Identification of the stay green genes responsible for water stress susceptibility

To determine which of the mutant genes in the *Gd1d2* genotype causes increased water stress susceptibility, near-isogenic lines with different combinations of *G*, *d1* and *d2* were subjected to a water deficit treatment (Table 4). After 10 d of treatment, relative water content decreased significantly in all genotypes, but this decrease was much more pronounced in lines carrying the stay green genotypes *d1d1d2d2* and *GGd1d1d2d2*, i.e. the homozygous combination of the recessive alleles *d1* and *d2* was responsible for increased water stress susceptibility. None of the lines carrying *G*, *d1* or *d2* alone or in combinations that do not cause the stay green trait had any effect on tolerance to drought.

Discussion

(G)d1d2 *responses to water deficit*

Lines of soybean carrying the stay green genotype *d1d1d2d2* are more susceptible to water deficit than their wild-type (i.e. normally senescing) near-isogenic counterparts. At a moderate soil water deficit, leaves of the stay green dehydrate irreversibly while comparable leaves of the wild type maintain a higher water potential and remain fresh.

The susceptibility of *(G)d1d2* to water deficit is not due to impaired regulation of stomatal aperture. Regardless of whether the stress treatment was applied to vegetative or to reproductive plants undergoing monocarpic senescence, stomatal conductance decreased to a similar extent in wild-type and stay green leaves. Moreover, the stomatal

Table 4. *Relative water content in unifoliate leaves of plants of wild-type cv. Clark and near isogenic lines carrying different combinations of* G, d1 *and* d2, *watered (control) and subjected to water stress*

ND: not determined. For each date, values followed by the same letter do not differ significantly at 5% (LSD test).

		Relative water content (%)		
		Day 0 (21 DAP)	Day 7 (28 DAP)	Day 10 (31 DAP)
Clark (*ggD1D1D2D2*)	Control	94.3 a	95.4 a	94.3 a
	Stressed		74.5 b	77.7 b
Gd1d2 (*GGd1d1d2d2*)	Control	95.8 a	94.8 a	95.2 a
	Stressed		77.5 b	52.4 c
d1d2 (*ggd1d1d2d2*)	Control	94.3 a	94.8 a	95.3 a
	Stressed		73.4 b	53.3 c
GGd1d1D2D2	Control	94.2 a	95.5 a	96.1 a
	Stressed		71.1 b	71.1 b
GGD1D1d2d2	Control	95.2 a	ND	94.5 a
	Stressed		ND	74.3 b
ggd1d1D2D2	Control	95.3 a	94.5 a	94.4 a
	Stressed		77.9 b	68.2 b
ggD1D1d2d2	Control	95.0 a	95.5 a	94.3 a
	Stressed		73.4 b	71.8 b

conductance of well-watered plants of *Gd1d2* growing outdoors during the normal growing season for soybeans can be even lower than that of wild type cv. Clark (Luquez and Guiamét, 2001). Exogenous applications of ABA reduce stomatal conductance in non-stressed controls, and in plants of *(G)d1d2* at the beginning of a drought period (day 3), indicating that the mutant responds normally to ABA. Closure of stomata in response to water deficit suggests that ABA accumulation is not impaired in *(G)d1d2*. The fact that exogenous applications of ABA have no effect protecting *(G)d1d2* leaves from accelerated dehydration under water deficit further substantiates the idea that impaired regulation of stomatal closure and/or a defect in ABA metabolism or response are not involved in the exacerbated water stress susceptibility of the mutant. Likewise, exogenous applications of ABA do not normalize the stay green phenotype of *(G)d1d2*, i.e. ABA does not cause Chl degradation in the mutant (Guiamét and Gianibelli, 1994).

Leaf dehydration in *(G)d1d2* against a background of normal regulation of stomatal conductance suggests that water absorption and flux through the roots might not be enough to cope with water loss in *(G)d1d2*, or to replenish leaf water content when stomata close at night. However, explants of *Gd1d2* dehydrate before abscission, very much like leaves attached to intact plants, indicating that changes at the leaf level may be involved in leaflet dehydration at the end of monocarpic senescence, and probably also in the increased susceptibility of the mutant to water stress.

Senescence and water balance

The comparison of lines carrying different combinations of *d1*, *d2* and *G* shows that increased susceptibility to water deficit is caused by the *d1d1d2d2* genotype, i.e. the combination of mutations that inhibits thylakoid and Rubisco degradation (Guiamét *et al*., 1990, 1991; Guiamét and Gianibelli, 1996). This indicates that there is a link between chloroplast preservation and water stress susceptibility. However, it is unlikely that retention of chloroplast components *per se* directly determines water deficit susceptibility. For example, the experiments with unifoliate leaves of 3-week-old plants started before symptoms of senescence (e.g. chlorophyll loss) became apparent in the wild type, and, therefore, well before the stay green trait is expressed in *Gd1d2*. Stay green mutants of other species retain chloroplast components without any apparent adverse effect on the water balance of leaves (Thomas and Smart, 1993), and stay green lines of sorghum and rice are actually more tolerant to water deficit than normally yellowing lines (Thomas and Howarth, 2000). If the stay green trait *per se* does not cause water deficit susceptibility, an alternative hypothesis might be that the primary action of the *d1d2* genotype is to cause premature cell death in leaves of the mutant, in response to a stress factor (e.g. water deficit) or during normal development of the plant (e.g. during senescence). Premature cell death would cause untimely cessation of chloroplast degradation, resulting in a type D stay green (Thomas and Howarth, 2000). However, *(G)d1d2* exhibits a completely stay green character in darkness without any visible symptom of leaf death, e.g. dehydration or decay (Guiamét and Gianibelli, 1994). Furthermore, dehydration of well-watered leaves of *G(d1d2)* occurs very late in senescence, whereas inhibition of chlorophyll and Rubisco degradation is already noticeable much earlier, even before the wild type has lost 50% of its chlorophyll, i.e. chloroplast preservation starts well before dehydration in *(G)d1d2* (Guiamét *et al*., 1990). This clearly argues that premature cell death could not be the cause of the stay green character of *(G)d1d2* and, by extension, of its susceptibility to water stress. A direct causal relationship between chloroplast preservation and stress susceptibility, or vice versa, is not apparent from these data.

Unlike *Gd1d2*, stay green lines of sorghum and rice are more tolerant to water deficit than their normally-senescing counterparts (Borrell *et al*., 2000*b*; Thomas and Howarth, 2000). In such species, selecting for plants that stay green during a drought period can be a plausible way to increase yield under drought (Borrell *et al*., 2000*b*). Stay green hybrids of sorghum seem to represent type A or type B stay greens, where leaf life span is prolonged either because the onset of senescence is delayed (type A) or the rate of leaf senescence is reduced (type B) (Thomas and Howarth, 2000). As a result, these hybrids retain more green leaf area at maturity (i.e. leaf life span is prolonged) when grown under terminal water deficit (Borrell *et al*., 2000*a*). By contrast, *(G)d1d1d2d2* may behave as a type C stay green, retaining chloroplast components, but probably not realizing its potential higher photosynthetic capacity in all environmental conditions, particularly under stress, and clearly not extending leaf life span. Moreover, while *Gd1d2* is strictly monocarpic, the stay green lines of sorghum show a tendency to perenniality, e.g. increased tillering (Duncan *et al*., 1981), and this reduced monocarpic influence on the vegetative parts of stay green grasses may contribute to the maintenance of green leaf area during a period of water deficit.

In summary, the stay green genotype *d1d1d2d2* shows increased susceptibility to water deficit compared to a near-isogenic wild-type line. Stress-response genes are up-regulated in senescing leaves and, in turn, senescence-associated genes are expressed under conditions of water deficit (Weaver *et al*., 1998). The expression of senescence-associated genes in stressed tissues, and the pleiotropic effects of *d1* and *d2* suggest that pathways involved in chloroplast disassembly and in the regulation of stress responses are intertwined and controlled by the same factors.

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