Acta Botanica Hungarica 45(3–4), pp. 399–407, 2003

ECOPHYSIOLOGICAL APPROACH TO STUDY OF THE ROLE OF CELL-BOUND PEROXIDASE FORMS IN WHEAT LEAVES OF DIFFERENT MORPHOLOGY

G. SARIYEVA and S. KENJEBAEVA

Lab. of Plant Growth and Resistance, Institute of Plant Physiology, Genetics and Bioengineering 45 Timiryazev str., Almaty, 480090, Kazakhstan; E-mail: gulmirasarijeva@web.de

(Received 16 May, 2003)

Extracellular peroxidase enzymes, both ionically – and covalently bound, have been implicated in the biochemical changes of cell wall and furthermore for regulation of leaf and root extension growth. However, the role of cell wall bound peroxidase in changes of mature leaf morphology is poorly understood. The aim of this study was to determine whether ionically and covalently bound peroxidase activities exhibit any relationships with drought-induced leaf rolling of wheat. An activity and thermostability of three wheat genotypes with different leaf rolling degree were studied. The results clearly show that two genotypes with leaf rolling trait exhibited significantly higher activity of both extracellular peroxidase fractions in comparison to non-rolling cv. 'Omskaya 9'. In addition, the significant genotypic differences were found in thermostability of ionically and covalently bound peroxidase. A 35 °C-treatment decreased the activity of ionically bound peroxidase (IBP) in non-rolling leaves of parent cultivar and enhanced the activity of IBP in rolling leaves of 'Alba' genotype. Under 45 and 55 °C-treatment the IBP was more stable in rolling leaves of both genotypes 'Otan' and 'Alba'. Despite to low activity of covalently bound peroxidase it was more stable after heating by 45 °C compare with ionically bound fraction. The heating of covalently bound peroxidase (CBP) extracts by 35 °C and 45 °C also has enhancing effect on its activity by both leaf rolling genotypes (on 16 and 40% by 'Otan' and 'Alba', respectively). At the same time the CBP activity of non-rolling genotype decreased significantly on 40%. Even after 55 °C-treatment the CBP of both rolling genotypes remained very stable, although there were further inhibition of CBP purificated from leaves of non-rolling genotype. Both wheat genotypes with inserted leaf rolling trait exhibited a much higher blue-green fluorescence emission than non-rolling leaves of parent cultivar 'Omskaya 9'. Thus, the molecular mechanism of leaf rolling regulation seems to include an adjustment of cell wall properties and probably involves an activity of cell wall bound peroxidase. Further investigation is expected to reveal the regulatory mechanism for leaf rolling in wheat.

Key words: activity, covalently bound peroxidase (CBP), ionically bound peroxidase (IBP), leaf rolling, thermostability, wheat

Abbreviations: IBP = ionically bound peroxidase, CBP = covalently bound peroxidase, WUE = water use efficiency, P_N = netto photosynthesis

0236–6495/$ 20.00 © 2003 Akadémiai Kiadó, Budapest

INTRODUCTION

During the summer, spring wheat vegetation is often subjected to periods of severe drought, and large reduction of leaves photosynthetic capacity is one of the symptoms of its influence. The depression in photosynthesis of wheat under field conditions is due to the combined effect of high temperature, high light + potentially harmful UV-radiation and water deficit. Plant response to all these factors occurs at a variety of organisational levels (Pearcy 1998, Lichtenthaler and Burkart 1999). One of them might be a change of leaf blade morphology as a diurnal response to increased air temperature and solar irradiance. Leaf rolling by some rice and maize genotypes coincided with an increased water use efficiency and osmotic adjustment under drought conditions (Dingkuhn *et al.* 1989, Premachandra *et al.* 1992). In our investigation, spring wheat genotypes with inserted leaf rolling trait displayed better performance for yield stability and productivity under severe drought. The two most leaf rolling wheat genotypes exhibited the best stomata regulation, a higher water use efficiency and the highest P_N-rates at a 40 °C heat stress than the non-rolling genotype (Lichtenthaler *et al.* 2002). At the same time, our understanding of the physiological mechanism of leaf rolling is still not well established.

The present study investigates a hypothesis that the temperature- and irradiance-induced leaf rolling of wheat occurs through changes in the biochemical properties of cell walls. It has been shown in several reports that the stiffening and rigidifying of cell walls under drought conditions could be responsible for expansion growth control of young leaves. The loss of cell wall elasticity resulting in growth stop is closely related to an increase in cell wall material monophenols such as ferulic acid, diferulic acid and p-coumaric acid (Fry 1979). The reactions of ferulic acid coupling with formation of diferulic acid catalyse peroxidase (EC 1.11.1.7) bound to the cell wall. There is a great deal of evidence indicating that cell wall peroxidase could be responsible for biochemical changes in the cell wall and furthermore for regulation of extension growth (Mijamoto *et al.* 1994, Bacon *et al.* 1997, Parvez *et al.* 1997, Thompson *et al.* 1997). However, the main object of most studies was still stress-induced growth response of young expanding leaves and roots. We have suggested that the extracellular peroxidase activity could be involved in drought-induced leaf rolling of mature flag leaves of wheat. Thus, the aim of our study was to investigate possible relationship between blue fluorescence intensity and activity of ionically and covalently bound cell wall peroxidase to determine an apoplastic peroxidase thermostability in non-rolling and rolling leaves of wheat.

MATERIAL AND METHODS

Plant material: 2 genotypes of spring wheat with leaf rolling trait named 'Otan' and 'Alba' were obtained from crossing of non-rolling cultivar 'Omskaya 9' on 'Grekum 476' by P. Bogdanova ED, Institute of Plant Physiology, Kazakhstan. 'Grekum 476' was the donor of leaf rolling genes Rl_1 and Rl_2. According to our classification, both genotypes with inserted leaf rolling trait 'Otan' and 'Alba' differ in leaf rolling diurnal degree. At midday, when solar irradiance peaks at about 1,400 μmol $m^{-2}s^{-1}$, genotype 'Otan' has the maximum degree of flag leaf rolling, that remains constant until 18 h in the evening (Sariyeva and Kenjebaeva 2000). The second genotype 'Alba' exhibits only a slow leaf rolling performance at the same time. The parent cultivar 'Omskaya 9' has no leaf-rolling trait and consequently cannot roll leaves during the day.

Fluorescence emission spectra: The plants for this research project were grown in a greenhouse (the Botanical Garden of the University of Karlsruhe, Germany) on a buffered mineral peat (TKS 2). The conditions in greenhouse were: 16 h photoperiod, 17/10 °C day/night temperature, 65–70% humidity, a maximum photon flux density of 700 μmol $m^{-2}s^{-1}$ performed by accessory light (Philips HLRG lamps, 400 W). For the measurements, fully developed flag leaves of 80-day-old plants were taken. Fluorescence emission spectra were recorded from 400–640 nm at an excitation wavelength of 340 nm using a Perkin Elmer LS–50 Luminescence Spectrometer. All measurements were made on the adaxial surface of 8–10 flag leaves per genotype.

Cell wall peroxidase activity: For the measurement of cell wall peroxidase activity, the plants were grown in the field with summer day/night temperatures of 30/20 °C and light at about 1,400 μmol $m^{-2}s^{-1}$ at noon. The intensity of solar irradiation was measured on flag leaves using a LICOR 300 portable porometer during the growing season between the end of May and the beginning of June. The mature, fully developed flag leaves of each genotype were taken at 12 h midday. Peroxidase extraction was done according to Gamburg *et al.* (1977). Fresh leaf material was homogenised in ice cold with 5 ml of Na-acetate-acetic buffer at pH = 4.5, containing 1 mol EDTA, 15 mmol $CaCl_2$ in ratio 1:5. The residue of cell walls material was washed 5 times with the same buffer to remove cytoplasmic peroxidase. To extract the ionically bound cell wall protein the cell wall material was incubated for 2 h in 2 ml 1 mol KCl at 4 °C at constant stirring. After centrifugation at 1,000 g for 10 min the supernatant was collected and used for the measurements of the ionically bound peroxidase activity. The cell wall pellet was washed twice with the same buffer by centrifugation and then was incubated overnight in 1 ml of 1% Triton X–100 in purification buffer by 4 °C. After centrifugation the supernatant was used for the measurements of the covalently bound peroxidase. Peroxidase activity

was measured by a spectrophotometric method in a 3-ml-cuvette. The reaction mixture consisted of a 50 mmol Na-acetate-acetic buffer (pH = 4.5), 3% (v/v) H_2O_2 and enzyme mixture. The reaction was started by the addition of the substrate o-dianisidine (50 µl of a 10 mg ml^{-1} solution in 96% ethanol). The progression of the reaction was followed by measuring the changes in absorbance at 470 nm for 1 min at 25 °C. Three enzyme extracts were prepared for each sample of tissue and assayed in quadruplicate. Results were calculated as relative units of the change in absorbance g^{-1} fresh weight min^{-1}. For the measurements of peroxidase thermostability, 0.2 ml of all extracts were heated for 2 h to the following temperatures: 35 °C, 45 °C, 55 °C and 65 °C and thereafter the peroxidase activity was determined as described above. As a control the activity of enzyme from the same extract assayed before heating at room temperature (25 °C) was taken.

Statistics: statistical analysis of the data was conducted by using ANOVA to establish significant differences between variances.

RESULTS

Applying a short wave with length 340 nm as the excitation source, blue fluorescence emission spectra, shown in Figure 1, were obtained. A typical fea-

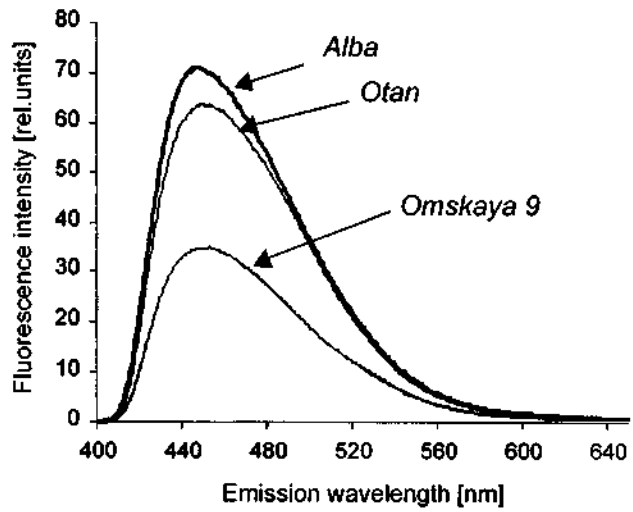

Fig. 1. Emission fluorescence spectra of flag leaf-rolling and non-rolling wheat genotypes. ('Omskaya 9' = non-rolling parents genotyp; 'Otan' = genotype with the most pronounced degree of leaf rolling; 'Alba' = genotype with the middle degree of leaf rolling. All measurements were made on adaxial surface of 8–10 flag leaves per genotype)

Table 1

An activity of extracellular peroxidase extracted from non-rolling and rolling leaves of wheat at flowering stage. The activity is expressed as absorbance g^{-1} fresh weight min^{-1} at 470 nm. Data are means ± SE, n = 12. * indicates significant differences from 'Omskaya 9' ($p < 0.05$)

Genotype	Ionically bound peroxidase	Covalently bound peroxidase
'Omskaya 9'	181 ±9.7	25 ±4.5
'Otan'	296 ±17*	50 ±9.1*
'Alba'	384 ±28*	74 ±8.3*

ture is the fact that both wheat genotypes with the leaf rolling trait exhibited a much higher blue-green fluorescence emission than non-rolling leaves of the parent cultivar 'Omskaya 9'. It is well established that peaks in the blue-green regions (440 and 520 nm) of the UV-induced fluorescence emission spectrum reflect secondary phenolic metabolites located and covalently bound to the cell walls (Lichtenthaler and Schweiger 1998, Takács *et al.* 2000, Csintalan *et al.* 2001). Among the phenols located in cell walls ferulic acid and diferulic acid are the main emitters of blue-green fluorescence. Since peroxidase activity is required for the formation on diferulates, we also studied an activity of both ionically and covalently bound péroxidase. Our results clearly show that both genotypes with leaf rolling exhibited a significantly higher activity of both peroxidase fractions (Table 1). In addition, in leaves of all genotypes the ionically bound peroxidase activity was much higher compared to the covalently bound activity.

It is well demonstrated that peroxidase of many plants displays a widely genetic and functional variability. These functional assignments are based on the catalytic properties, expression profiles, localisation and characteristics of peroxidase-expressing genes (Hiraga *et al.* 2001). Thermostability has been known as one of the important catalytic properties of peroxidase and many authors have reported that peroxidase can be established as an enzyme with very high variation of thermostability in response to a broad temperature interval (Sarsenbayev *et al.* 1983, Ivakin and Grushin 1986).

Based on this point of view we investigate the thermostability of cell bound peroxidase extracted from non-rolling and rolling leaves of wheat. The heating of extracts from flag leaves to 35 °C decreased the IBP activity by the non-rolling parent cultivar 'Omskaya 9', enhanced on 20% the activity by the slow leaf rolling 'Alba' and did not have an effect on the IBP activity by genotype 'Otan' with most pronounced leaf rolling degree (Fig. 2A). An increase in heating the temperature to 45 °C inhibited the IBP by 35% as compared to the control (an activity of IBP under room temperature) by 'Alba' and non-rolling parent, although the IBP activity in rolling leaves of 'Otan' remained un-

changed. Commonly, the IBP extracted from flag leaves of both genotypes with leaf rolling trait were more stable in temperature interval from 35 °C to 55 °C.

Despite the low activity of covalently bound peroxidase, it was more stable after heating to 45 °C compared with ionically bound fraction (Fig. 2B). The heating of CBP extracts to 35 °C and 45 °C also has an enhancing effect on its activity for both leaf-rolling genotypes (on 16 and 40% by 'Otan' and 'Alba', respectively). At the same time, the CBP activity of non-rolling genotype decreased significantly by 40%. Even after 55 °C-treatment, the CBP of both rolling genotypes remained very stable, although there was further inhibition of CBP purified from leaves of non-rolling genotype.

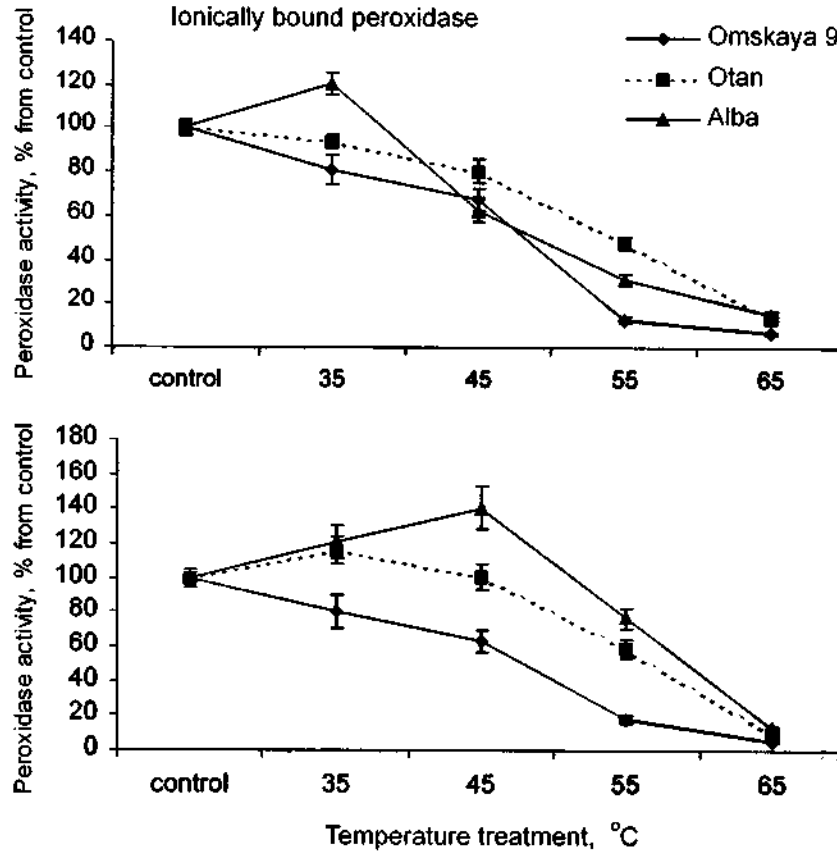

Fig. 2. Activity of ionically bound peroxidase (top) and covalently bound peroxidase (bottom) from flag leaves of three wheat genotypes with different degree of leaf rolling. The activity was measured after enzyme extracts heating for 2 hours to different temperatures. As a control the activity of enzyme assayed at 25 °C (room temperature) was taken. Data are means ± SE, n = 12

DISCUSSION

The fact that an intensity of blue-green fluorescence is closely related to the accumulation of flavonoids in epidermis cells demonstrates that the method of blue-green fluorescence could be successfully used for screening biochemical compounds that absorb UV-irradiance. Therefore the highest intensity of blue-green fluorescence suggested that two wheat genotypes with leaf rolling trait accumulate more flavonoids in their epidermal cells in response to high solar irradiation and drought conditions.

In addition to more intensive blue-green fluorescence, the genotypes with leaf rolling exhibited a higher activity of cell wall bound peroxidase. This fact can also indicate the greater amount of flavonoids in the cell walls of rolling leaves, since an extracellular peroxidase activity closely correlates with an accumulation of ferulates and diferulates in the cell walls of many plants (MacAdam and Grabber 2002). The accumulation of flavonoids in epidermal cells, absorbing a greater part of potentially harmful UV-irradiance, protects the mesophyll cells by decreasing chlorophyll excitation (Havaux and Kloppstech 2001). On the other hand, both IBP and CBP are commonly thought to play a key role in rigidifying cell walls and in controlling temporary and spatial growth rate (Thompson *et al.* 1997). In our case this fact could be interpreted in relation to diurnal changes of leaf blade morphology displayed by wheat genotypes with leaf rolling trait. Perhaps one of the leaf rolling mechanisms could be stiffening and rigidifying of cell walls induced by enhanced activity of cell wall bound peroxidase. As to differences in the activity of IBP and CBP, it should be noted that many authors reported a greater activity of IBP compared to CBP in growing leaves and coleoptiles (Ikegawa *et al.* 1996, Gonzalez and Rojas 1999). It was proposed that IBP plays a more important role in producing diferulates in cell walls. We can also suggest that IBP extracted from all genotypes of leaves is more responsible for the accumulation of phenols in the cell walls of mature flag leaves as compared to CBP.

In addition to a high level of common cell wall bound peroxidase activity extracted from rolling leaves, we observed a high thermostability of both enzyme fractions in a wide temperature interval. Both genotypes with leaf rolling trait were characterised by stable IBP and extremely stable CBP in the range of 35–45 °C (Fig. 2A–B). Our study does not enable us to identify a course for increase in both extracellular peroxidase activities after heating to 35 °C and 45 °C. The increase in activity of soluble peroxidase in response to drought was reported previously as a marked feature of drought and heat resistant genotypes of wheat and tomatoes (Sarsenbayev *et al.* 1983, Ivakin and Grushin 1986). But there is no information about the thermostability of cell wall bound peroxidase. The loss of both IBP and CBP activities after 55 °C and 65 °C tem-

perature treatment should be due to enzyme inactivation. The clear genotypic differences in activity and thermostability of both IBP and CBP demonstrated here are probably based on different expression levels of peroxidase genes. Relatively rapid changes in leaf rolling degree demonstrated by both genotypes during a day indicate an availability of a highly regulated mechanism at the molecular level. Beside extracellular peroxidase, other enzymes located in the cell wall could be involved in this mechanism. They are xyloglucan endotransglycosylase, which is responsible for the loosening of polysaccharides matrix, protein expansin, H^+-ATPases. These polymers activities can control wall pH, ion concentration (Wu and Cosgrove 2000), which in turn may modulate both the activity of wall enzymes and the physical properties of the wall matrix.

Thus, the molecular mechanism of leaf rolling regulation seems to include an adjustment of cell wall properties and probably involves an activity of cell wall bound peroxidase. Further investigation is expected to reveal the regulatory mechanism for leaf rolling in wheat.

REFERENCES

Bacon, M. A., Thompson, D. S. & Davies, W. I. (1997): Can cell wall peroxidase activity explain the leaf growth response of Lolium t. L. during drought? – *J. Exp. Bot.* **317**: 2075–2085.

Csintalan, Z., Tuba, Z., Takács, Z. & Laitat, E. (2001): Responses of nine bryophyte and one lichen species from different microhabitats to elevated UV-B radiation. – *Photosynthetica* **39**(2): 317–320.

Dingkuhn, M., Cruz, R. T., O'Toole, J. C. & Dorfling, K. (1989): Net photosynthesis, water use efficiency, leaf water potential and leaf rolling as affected by water deficit in tropical upland rice. – *Aust. J. Agric. Res.* **40**: 1171–1181.

Fry, S. C. (1979): Phenolic compounds of the primary cell wall and their possible role in the hormonal regulation of growth. – *Planta* **146**: 343–351.

Gamburg, K. S., Podolyakina, L. A. & Sitneva, W. M. (1977): The activity of peroxidase and indolilaceticacidoxidase in suspension cultures of tabak and soybean tissues. – *Rus. J. Plant Physiol.* **24**(3): 542–548.

Gonzalez, L. F. & Rojas, M. C. (1999): Role of wall peroxidases in oat growth inhibition by DIMBOA. – *Phytochemistry* **50**: 931–937.

Havaux, M. & Kloppstech, K. (2001): The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in Arabidopsis npq and tt mutants. – *Planta* **213**: 953–966.

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y. & Matsui, H. (2001): A large family of class III plant peroxidases. – *Plant Cell Physiol.* **42**(5): 462–468.

Ikegawa, T., Mayama, S., Nakayashiki, H. & Kato, H. (1996): Accumulation of diferulic acid during the hypersensitive response of oat leaves to Puccinia coronata f. sp. avenae and its role in the resistance of oat tissues to cell wall degrading enzymes. – *Physiol. Mol. Plant Pathol.* **48**: 245–255.

Ivakin, A. P. & Grushin, A. A. (1986): Thermostability of peroxidase in tomato cultivars differing in heat resistance. – *Rus. J. Plant Physiol.* **33**(2): 226–234.

Lichtenthaler, H. K. & Burkart, S. (1999): Photosynthesis and high light stress. – *Bulg. J. Plant Physiol.* **25**: 3–16.

Lichtenthaler, H. K. & Schweiger, J. (1998): Cell wall bound ferulic acid, the major substance of the blue-green fluorescence emission of plants. – *J. Plant Physiol.* **152**: 272–282.

Lichtenthaler, H. K., Sariyeva, G., Kenjebaeva, S. S. & Polimbetova, F. A. (2002): *Differences in photosynthesis to temperature stress in non-rolling and leaf-rolling spring wheat genotypes.* – Abstracts, 13 FESPP, Crete, Greece, 2002, p. 454.

MacAdam, J. W. & Grabber, J. H. (2002): Relationship of growth cessation with the formation of diferulate cross-links and p-coumaroylated lignins in tall fescue leaf blades. – *Planta* **215**: 785–793.

Mijamoto, K., Ueda, J., Takeda, S., Ida, K., Hoson, T., Masuda, Y. & Kamisaka, S. (1994): Light-induced increase in the contents of ferulic and diferulic acids in cell walls of Avena coleoptiles: its relationship to growth inhibition by light. – *Physiol. Plant.* **92**: 350–355.

Parvez, M. M., Wakabayashi, K., Hoson, T. & Kamisaka, S. (1997): White light promotes the formation of diferulic acid in maize coleoptile cell walls by enhancing PAL activity. – *Physiol. Plant.* **99**: 39–48.

Pearcy, R. W. (1998): *Acclimation to sun and shade.* – In: Raghavendra, A. S (ed.): Photosynthesis. A Comprehensive Treatise. Cambridge University Press, Cambridge, pp. 250–263.

Premachandra, G. S., Saneoka, H., Fujita, K. & Ogata, S. (1992): Osmotic adjustment and stomatal response to water deficits in maize. – *J. Exp. Bot.* **256**(43): 1451–1456.

Sariyeva, G. S. & Kenjebaeva, S. S. (2000): The effect of drought on the growth and water status of rolling flag leaves by wheat. – *Proceedings of Sciences Academy Kazakhstan* **6**: 48–56.

Sarsenbayev, K. N., Titov, N. N., Mezenceva, N. I. & Polimbetova, F. A. (1983): The activity of soluble and bound peroxidases of maturing grain of spring wheat. – *The Agricult. Biol.* **11**: 32–36.

Takacs, Z., Lichtenthaler, H. K. & Tuba, Z. (2000): Fluorescence emission spectra of dessication tolerant cryptogamic plants during a rehydration-dessication cycle. – *J. Plant Physiol.* **156**: 375–379.

Thompson, D. S., Wilkinson, S., Bacon, M. A. & Davies, W. J. (1997): Multiple signals and mechanisms that regulate leaf growth and stomatal behaviour during water deficit. – *Physiol. Plant.* **100**: 303–313.

Wu, Y. & Cosgrove, D. J. (2000): Adaptation of roots to low water potentials by changes in cell wall extensibility and cell wall proteins. – *J. Exp. Bot.* **51**(350): 1543–1553.