Two Mutants Affecting Adaptative Responses to Abiotic Stresses in Barley Seedlings

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Abstract: Two novel mutants which affect the adaptative responses of barley seedlings to different abiotic stresses are described. They allow us to explore some aspects of adaptative phenomena that are little known in higher plants. One of these mutants corresponds to a nuclear gene which under certain circumstances in the wild type barley induces additional ethylene production in the seedling roots. This mechanism seems to be involved in inducing a negative hydrotropic growth of the roots, a phenomenon that we interpret as a response avoiding waterlogging. The other mutant corresponds to a plastid encoded gene which is involved in photosystem I and II stability and, probably, indirectly affects the acclimation of the seedlings to higher temperatures, a fact which seems to occur through the control of unsaturation/saturation levels of the thylakoid membrane fatty acids.

Keywords: abiotic stress; barley; root behaviour; temperature sensitivity

Two novel mutants, either of them affecting the response of barley seedlings to two important abiotic stresses such as waterlogging and high temperatures, are analysed, bearing in mind that they can be useful tools to investigate the highly complex mechanisms by which plants can adapt themselves to adverse environmental conditions.

One of these mutants was identified in hydroponics (Martínez *et al*. 2004) by the sandwich method of Myhill and Konzak (1967). In those particular conditions, mutant roots did not show the growth pattern with windings and turnings usually observed on wild type roots before they submerged. It was also demonstrated that this root behaviour was controlled by a semi-dominant nuclear gene (Martínez *et al*. 2004). The unusual root growth was postulated to be associated with diminished ethylene production. Indeed, ethylene diffused from mutant roots was significantly lower than that from wild type roots and, in addition, one experiment with the ethylene receptor antagonist silver ion (Drew *et al*. 1981) supported that hypothesis (see Martínez *et al*. 2004). Interestingly, no phenotypic differences were noticed between mutant and wild type roots when they grew freely inside a humid chamber. In the present work, we attempt to distinguish if the observed differences in ethylene diffused from wild type and mutant barley roots are either constitutive or differentially induced by the growth conditions of the sandwich method. At this respect the ethylene production by wild type and mutant roots was measured in two growing conditions, sandwich method and humid chambers. Additionally, in order to study the physiological basis of this root behaviour, the responses of wild type and mutant roots to different

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doses of ethylene related plant growth regulators (PGRs) were analysed.

The other mutant analysed here is a high temperature-sensitive one (Prina *et al.* 2000) that was previously described as a *viridis* type and showed maternal inheritance (Prina 1996). Wild type and mutant seedling responses to different environments were studied by measuring, on their first leaf blade, the content of chlorophylls *a* and *b*, carotenoids and water as well as the thylakoid protein composition and the saturation degree of the thylakoid membrane fatty acids.

MATERIALS AND METHODS

Plant material. Barley (*Hordeum vulgare* L.) seedlings were used in all assays. Root studies were carried out on seedlings of a root mutant (Martínez *et al*. 2004) and of the wild type control. Biochemical analyses of the first leaf blade were done on seedlings of the high temperature sensitive *viridis* mutant CL3 (Prina 1996), and of the wild type control. In this case, the second generation seedlings of reciprocal crosses were also analysed in some of the experiments.

Growth conditions. For most of the assays seedlings were grown in hydroponics by the sandwich method (Myhill & Konzak 1967). For some of the assays seedling roots were grown inside 15-ml Pyrex test tubes that behaved as humid chambers (Martínez *et al*. 2004).

For root analyses, seedlings were grown during seven days in a growth chamber (Conviron EF7 model) with a photoperiod of 14 h at 18°C. The following ethylene related plant growth regulators (PGRs) were added at sowing in the sandwich method assays: aminocyclopropane carboxylic acid (ACC, ICN Biomedicals Inc.); silver ions (Ag^+, added as silver thiosulphate); and aminoethoxyvinyl glycine (AVG, Retain®, NUFARM, New Zealand).

For biochemical analyses of the first leaf blade environmental conditions were as follows: assays of Figures 6, 7 and 8 were done in a growth chamber (SANYO Versatile Environmental Test Chamber), with a photoperiod of 18 h of light (100 μE/m^2s from white fluorescent tubes) at 18 or 32°C; assay of Figure 9 was carried out in a growth chamber (Conviron S10H) with a photoperiod of 18 h (100 μE/m^2s from white fluorescent tubes and incandescent lamps); for the assay of Figure 10 two different environments were used: (I) in the greenhouse, with natural light and temperatures ranging from 15 to 25°C and (II) in a growth chamber (SANYO) at 32°C and 18 h of light (100 μE/m^2s from white fluorescent tubes).

Ethylene quantification. The ethylene produced by wild type and mutant roots was measured by gas chromatography as described earlier (Martínez *et al*. 2004).

Biochemical analysis of the first leaf blade. Seedlings of CL3 and of its wild type control were subjected to the analysis of the following biochemical characteristics:

Pigment content: Chlorophylls *a* and *b* and carotenoid pigments were analysed in a Beckman DB-G spectrophotometer, according to MacLachlan and Zalik (1963) as described in Prina *et al.* (2003).

Thylakoid membrane proteins: Fractionation of thylakoid membranes and SDS-PAGE of their proteins were done based on Camm and Green (1980). Western blotting was performed according to ECL Western Blotting Analysis System by Amersham. For immunodetection of proteins, antisera raised in rabbit against highly purified D1 (Volker & Barkan 1995) and PSI (from barley, recognises PSI-A/B, -D, -E, -L, -H, -C) were used, which were kindly provided by Prof. A. Barkan (University of Oregon) and by Prof. H.V. Scheller (Royal Veterinary and Agricultural University, Denmark), respectively.

Water content: Water content was determined from differences between the fresh and the dry weight of the first leaf blade after 4½ hours at 105°C and was expressed as percentage of fresh weight.

Thylakoid membrane lipids: Lipid separation was done according to Pearcy (1978), but using HPLC instead of gas chromatography (GC). Lipids were quantified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Statistical analysis. Estimations of statistically significant changes in response to the different treatments and/or environments were done by Student's *t*-test for unpaired samples.

RESULTS AND DISCUSSION

A mutant lacking root tropic response to submergence

Ethylene production by wild type and mutant roots in different growth conditions. In Figures 1A and 1B marked morphological differences can be observed between wild type roots and mutant roots when

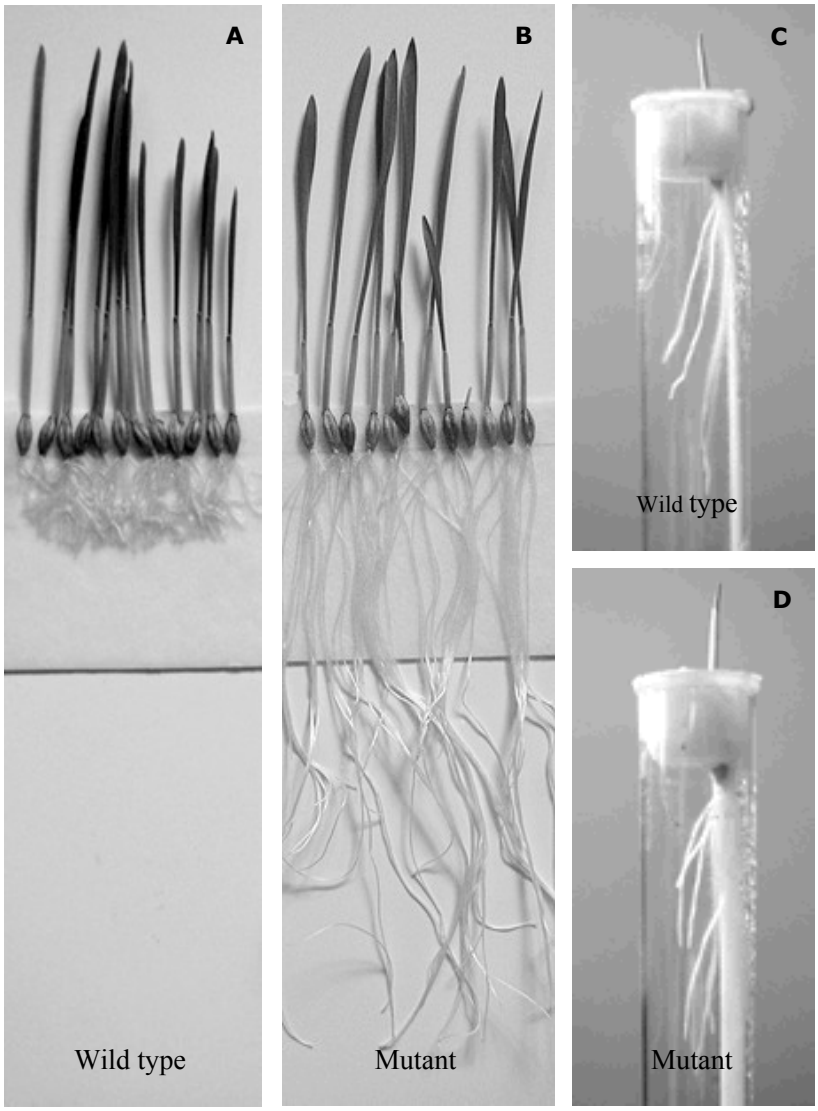

Figure 1. Wild type and root mutant seedlings were grown during seven days in water by the sandwich method (A and B) or inside humid chambers (C and D)

seedlings were grown in hydroponics by the sandwich method. Estimations of ethylene released by roots grown in these conditions confirmed previous results (Martínez *et al*. 2004), that means ethylene produced by mutant roots was significantly lower when compared with that of wild type roots (Figure 2). On the other hand, inside humid chambers where roots grew freely without any physical contact wild type roots and mutant roots were morphologically similar (Figures 1C, 1D) and they also had a similar ethylene production (Figure 2). Moreover, the amount of ethylene produced by both genotypes under these conditions was similar to that produced by mutant roots grown by the sandwich method (Figure 2). These results suggest that wild type barley roots are able to increase ethylene production in response to the special conditions given by the sandwich method. This response was observed to be related with a tropic response that results in an erratic growth pattern, with windings and turnings, displayed by wild type roots before they submerged. Several associated morphological changes such as root shortening and others not described here, e.g. root thickening and changes in root hair length and distribution, were also observed. This particular behaviour of wild type roots could be interpreted as an adaptative response of barley seedlings to flooding conditions.

Effects of ethylene related PGRs on the length of wild type and mutant roots. In order to further investigate the physiological basis of that root growth response, we assayed diverse ethylene related PGRs and their effects were quantified by measuring the length of the primary roots after seven days.

Addition of the ethylene precursor aminocyclopropane carboxylic acid (ACC, Tanimoto *et*

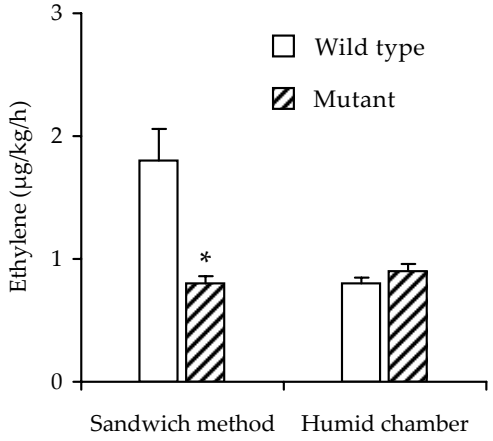

Figure 2. Ethylene production in wild type and mutant roots. Ethylene released from roots was measured on 7 d old seedlings grown in water by the sandwich method or in humid chambers. Results shown are means ± SE of three independent assays. *$P < 0.025$ compared with the wild type control

al. 1995), at 500µM concentration, caused a significant decrease of root length in both genotypes (Figure 3). These results suggest that an excess of ethylene may display inhibitory effects on root lengthening.

On the other hand, when the inhibitor of ethylene synthesis aminoethoxyvinyl glycine (AVG, Heidstra *et al*. 1997) was supplied at 1µM (Figure 4), it did not modify the mutant root length, but it induced a significant increase in the control root length. At higher concentrations, from 10 to 500 µM, AVG had root growth inhibitory effects on both genotypes (Figure 4).

Previously, after treatments with silver ions (Ag^+), ethylene receptor blockers (Drew *et al*. 1981), a marked increase in the wild type root length was observed while the mutant root length was not modified (Martínez *et al*. 2004). In the present work a more complete dose-response curve to Ag^+ was performed (Figure 5). At the two lower doses, Ag^+ treatments did not modify the mutant root length, but from 2.5mM concentration and thereafter a dose-dependent root shortening was observed. On the other hand, wild type root lengthening was stimulated with Ag^+ doses from 0.10 to 2.50mM, reaching the maximum length with 1.00mM Ag^+ dose. Higher doses of Ag^+ reduced the wild type root length in the same manner as they did in mutant roots.

It is interesting to remark the similarities in the two response curves observed after the addition of either an ethylene synthesis inhibitor (Figure 4) or an ethylene receptor blocker (Figure 5). All results confirm the involvement of ethylene in the different responses of mutant and wild type roots when they are grown by the sandwich method. They also suggest that a certain minimal endogenous

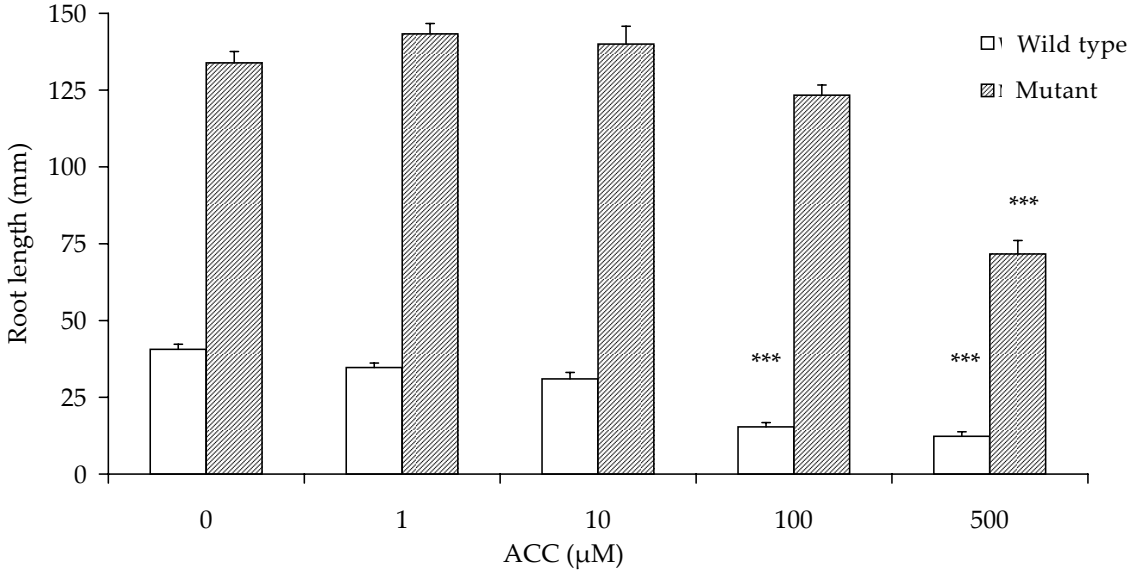

Figure 3. Effects of aminocyclopropane carboxylic acid (ACC) on root length of wild type and root mutant seedlings. Root length was recorded from seedlings grown during seven days in hydroponics by the sandwich method; the indicated doses of ACC were added at sowing. Data shown are the means ± SE of 10 to 15 roots. ***$P < 0.0001$ compared with the corresponding water control (0µM ACC)

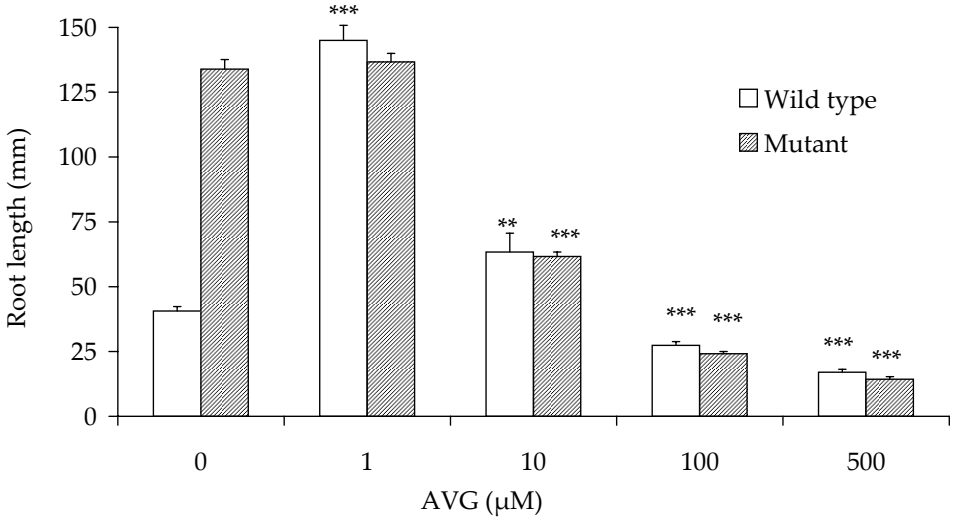

Figure 4. Effects of aminoethoxyvinyl glycine (AVG) on the root length of wild type and root mutant seedlings. Root length was recorded from seedlings grown during seven days in hydroponics by the sandwich method; the indicated doses of AVG were added at sowing. Data shown are the means ± SE of 10 to 15 roots. $^{**}P < 0.001$ and $^{***}P < 0.0001$ compared with the corresponding water control (0μM AVG)

ethylene action is necessary for root lengthening, but the root growth process is inhibited above a certain ethylene level. We hypothesised that this happened in wild type roots when an additional ethylene production was induced by the special wet conditions created above the water surface by the sandwich method.

A temperature sensitive mutant

Pigment analysis. Results of pigment content in the first leaf blade of wild type and CL3 seedlings grown at 32°C are presented in Figure 6. Similar results from reciprocal crosses (data not shown) confirmed the maternal inheritance of these pig-

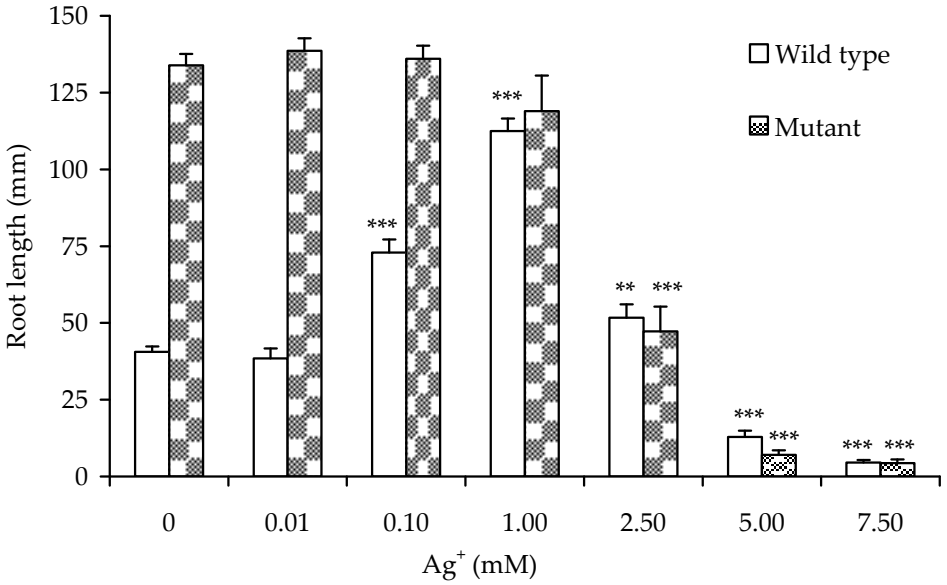

Figure 5. Effects of silver ions (Ag^+) on the root length of wild type and root mutant seedlings. Root length was recorded from seedlings grown during seven days in hydroponics by the sandwich method; the indicated doses of Ag^+ were added at sowing. Data shown are the means ± SE of 10 to 20 roots. $^{**}P < 0.001$ and $^{***}P < 0.0001$ compared with the corresponding water control (0mM Ag^+)

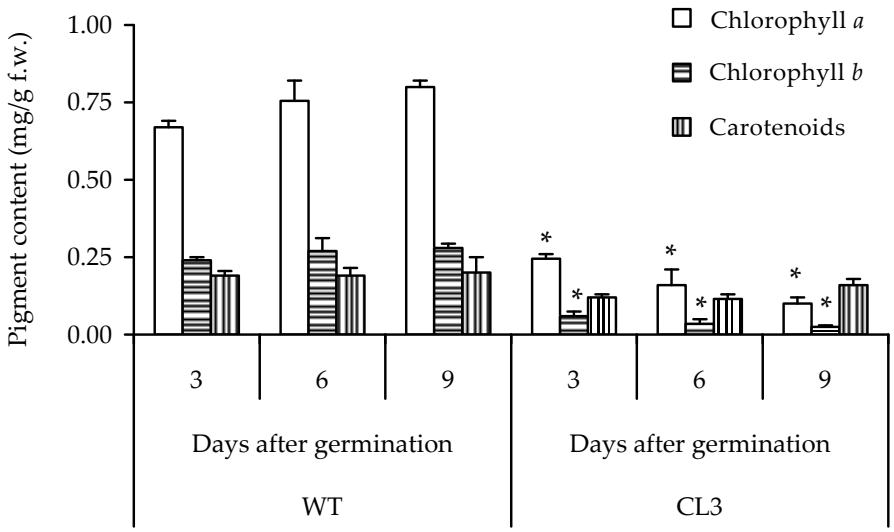

Figure 6. The first leaf blade pigment content at 32°C. Wild type (WT) and *viridis* mutant (CL3) seedlings were grown in tap water by the sandwich method in a growth chamber at 32°C. Pigment content was determined at 3, 6 and 9 days after germination as described in Materials and Methods. Results shown are means ± SE of two independent assays. *$P < 0.010$ with respect to equivalent data of WT chlorophyll *a*; *$P < 0.050$ with respect to equivalent data of WT chlorophyll *b*

ment content characteristics (Prina 1996). In CL3 seedlings the chlorophyll content was significantly lower than that of the wild type and diminished in time while carotenoids maintained a constant level (Figure 6) indicating that the observed chlorophyll diminution was not due to damage originating from a deficient carotenoid content.

Water content studies. The first leaf blade water content of wild type and CL3 seedlings grown at 32°C is represented in relation with time in Figure 7. It can be said that a constant temperature of 32°C is markedly lethal for seedlings carrying CL3 cytoplasm, which suffered a dramatic loss of water after 9 days.

Thylakoid membrane protein analysis. SDS-PAGE of thylakoid proteins of wild type and CL3 seedlings grown at 18 or 32°C is shown in Figure 8. After 9 days at 32°C, the pattern of thylakoid membrane proteins was completely altered in the CL3 first leaf blade. The corresponding Western blot with D1 antiserum (Figure 9) showed that D1 protein was not detected in CL3 by day 9. On the other hand, at 18°C (Figures 8 and 9), slighter differences were observed between CL3 and the control. Western blotting with antiserum against PSI proteins revealed the presence of PSI-A/B, PSI-D, PSI-C and PSI-E in the first leaf blade of wild type seedlings at both temperatures (Figure 10). In CL3 leaves, at 18°C, PSI-D appeared as a weak band at day 3 and it became stronger and of similar intensity to that of the control by day 9, meanwhile, PSI-E and PSI-C were not observed either at day 3 or day 6, but they appeared as very weak bands at day 9. At 32°C, in CL3, PSI-D was observed as a weaker band than at 18°C and PSI-E and C were not observed either at day 3, 6 or 9.

Thylakoid membrane lipid analysis. In relation to adaptative responses, the most striking differences between CL3 mutant and wild type seedlings were observed in the fatty acid saturation degree of the main thylakoid membrane lipids: mono-galactosyl-diacyl-glycerol (MGDG), di-galactosyl-diacyl-glycerol (DGDG) and phosphatidyl-glycerol (PG). In CL3 seedlings the proportion of linoleic acid did not show any marked differences between environment I and II (Figure 11) and similar percentages of linoleic acid were also observed for wild type seedlings in environment I. Curiously, in environment II, at 32°C, the wild type presented much higher percentages of linoleic acid (18:2) in the main thylakoid membrane lipids than in environment I (Figure 11). This occurred with a concomitant decrease in the more unsaturated linolenic acid (18:3) (data not shown).

Greater lipid saturation might be expected to confer thermal stability to membranes because of its higher melting temperatures (Pearcy 1978) and has been observed to play an important role

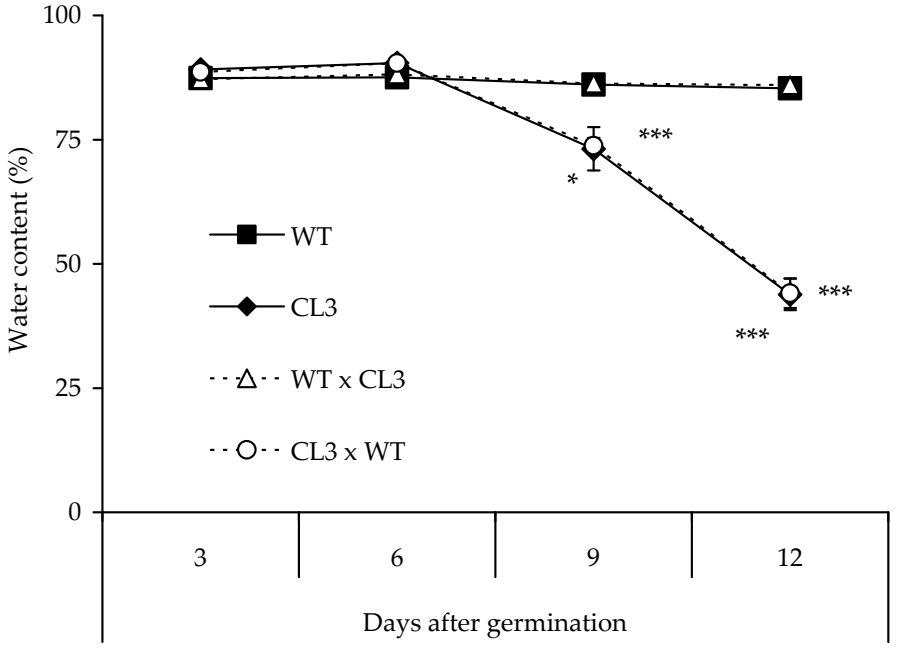

Figure 7. The first leaf blade water content. Results shown represent the leaf water content determined at different moments after germination as indicated and expressed as percent of fresh weight. Data are means ± SE of eight blades of wild type (WT), *viridis* mutant (CL3), and the reciprocal crosses of both (WT × CL3 and CL3 × WT). *$P < 0.025$ and ***$P < 0.001$ with respect to the corresponding WT values

at the chloroplast membrane level in improving thermal tolerance (Raison *et al*. 1982; Murakami *et al*. 2000; Alfonso *et al*. 2001; Iba 2002). From the present results it can be stated that, under certain circumstances, wild type seedlings seem to display an acclimation response, which is not functional in CL3 seedlings. All observations suggest that CL3 has a delayed assembly of PSI components and,

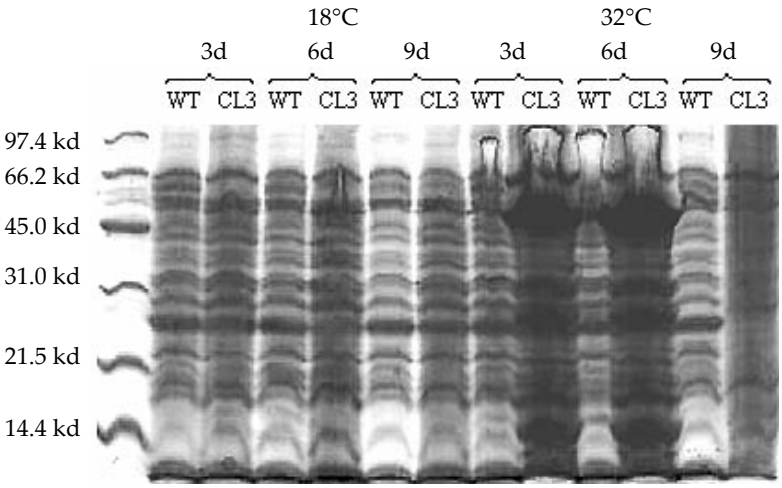

Figure 8. SDS-PAGE of thylakoid membrane proteins

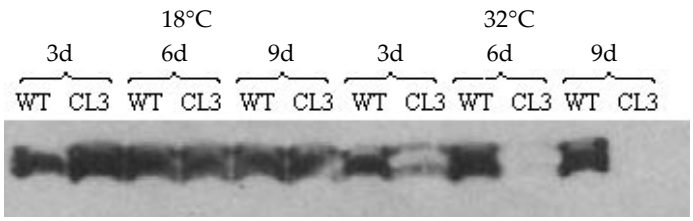

Figure 9. Western blot with D1 antiserum

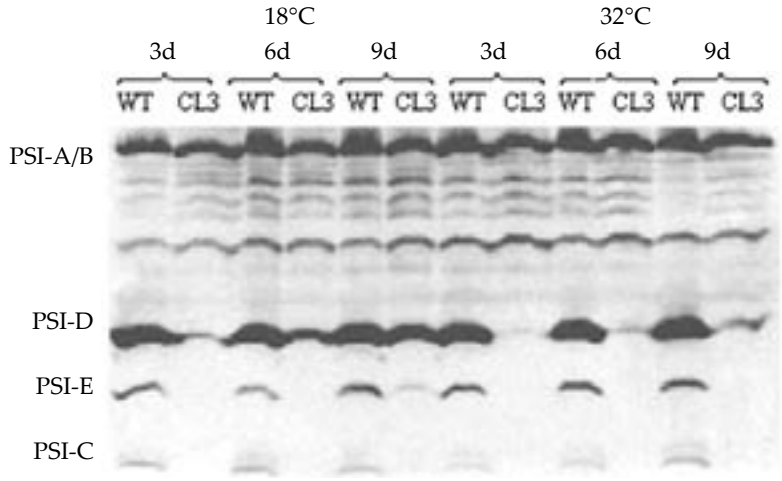

Figure 10. Western blot with PS1 antiserum

as a consequence, they could be easily damaged by environmental stresses. On the other hand, the observed decrease in PSII probably resulted from photoinhibition caused by a deficient PSI. Alfonso *et al*. (2001) pointed out that any factor affecting photosynthesis might ultimately influence the activity of fatty acid desaturases and then the capacity to adapt to environmental stresses. It is probable that deficiencies in the CL3 photosynthetic machinery would be responsible for its lack of CL3 fatty acid saturation response and that a chloroplast signal would be necessary for such response. Tanaka *et al*. (2000) proposed several models for acclimation mechanisms of the photosynthetic machinery to high temperature in *Chlamydomonas reinhardtii*, which involve different nuclear-chloroplast interactions.

Even though much more investigations are needed to clarify the mechanisms which are defective in the two mutants presented here, actual results call attention to potentially important adaptative phenomena by which wild type barley seedlings respond to different abiotic stresses, i.e. by changing root growth, morphology and tropism under

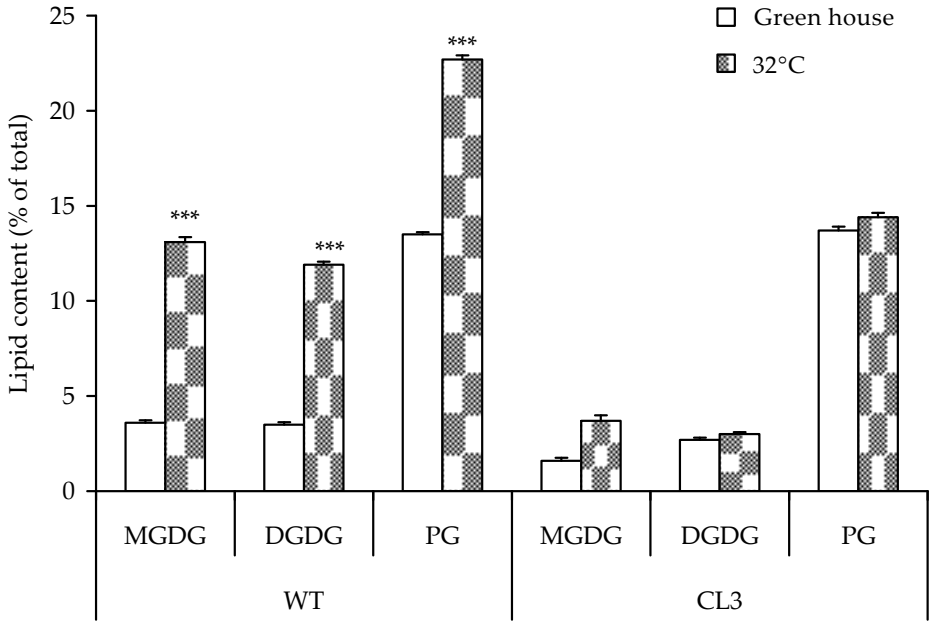

Figure 11. Percentage of linoleic acid (18:2) in the main thylakoid membrane lipids (MGDG, DGDG and PG). The content of 18:2 fatty acid is presented as percentage of the main thylakoid membrane lipids. Data are means ± SE of three replications. ***$P < 0.001$ compared with data obtained in the greenhouse

increasing wet conditions or by acclimation of the thylakoid membrane fluidity in order to face higher temperatures.

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Souhrn

Martínez A. E., Landau A., García P. T., Polenta G., Arias M. C., Murray R., Pensel N., Prina A. R. (2005): **Dva mutanty ovlivňující adaptační reakce na abiotické stresy u mladých rostlin ječmene**. Czech J. Genet. Plant Breed., **41**: 1–10.

V práci jsou popsány dva nové mutanty, které ovlivňují adaptační reakce klíčních rostlin ječmene na různé abiotické stresy. Tyto mutanty dovolují zkoumat některé aspekty adaptace, které jsou dosud u rostlin málo známy. Jeden z těchto mutantů má charakter jaderného genu, který za určitých okolností u divokého ječmene indukuje dodatečnou produkci etylenu v kořínčích rostlin. Zdá se, že tento mechanismus je začleněn do indukce negativního hydrotopického růstu kořenů, který se dá interpretovat jako reakce vylučující zavodnění kořenového systému. Druhý mutant odpovídá genu, který je zakódován v plastidu a který je zahrnut do stability fotosystému I a II. Pravděpodobně nepřímo tento gen ovlivňuje přizpůsobení rostlin vyšším teplotám, což má souvislost s řízením úrovně nenasycení/nasycení thylakoidní membrány mastnými kyselinami.

Klíčová slova: abiotický stres; ječmen; chování kořenů; citlivost na teplo

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