Difference of Physiological Characters in Dark Green Islands and Yellow Leaf Tissue of *Cucumber mosaic* Virus (CMV)-Infected *Nicotiana tabacum* Leaves

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Dark green islands (DGIs) are a common symptom of plants systemically infected with the mosaic virus. DGIs are clusters of green leaf cells that are free of virus but surrounded by yellow leaf tissue that is full of virus particles. In *Cucumber mosaic virus* (CMV)-infected *Nicotiana tabacum* leaves, the respiration and photosynthesis capabilities of DGIs and yellow leaf tissues were measured. The results showed that the cyanide-resistant respiration was enhanced in yellow leaf tissue and the photosynthesis was declined, while in DGIs they were less affected. The activities of the oxygen-scavenging enzymes catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in infected leaves were significantly higher than those in the healthy leaves, and the enzyme activities in DGIs were always lower than in the yellow leaf tissues. Reactive oxygen species (ROS) staining showed that the hydrogen peroxide content in yellow leaf tissues was apparently higher than that in DGIs, while the superoxide content was on the contrary. Formation of DGIs may be a strategy of the host plants resistance to the CMV infection.

Key words: CMV, Cyanide-Resistant Respiration, Dark Green Islands

Introduction

Virus infection is a kind of bio-stress, which is one of the most important limitations to crop productivity. Dark green islands (DGIs) had been a focus of morphological and cytological studies for a long time, even before the nature of viruses was known (Allard, 1914). Cells within DGIs are free of viral RNAs and proteins (Atkinson and Matthews, 1970). These cells also demonstrate resistance to super-infection by the original and closely related viruses but are susceptible to infection by unrelated viruses (Fulton, 1951). DGIs have a phenotype similar to healthy tissue, and DGIs formation is a developmentally predictable event that occurs at random sites across a leaf. Typically, DGIs encompass more than one cell layer and contain more cells than can be accounted for, if DGIs were the product of a single cell's division (Atkinson and Matthews, 1970). Yellow leaf tissue is virus-infected tissue, chlorophyll synthesis happens in serious delays than in the thinner DGIs. According to the great differences in the appearance of green and yellow tissue, there must have been great differences in their physiological processes.

Many studies have proved that alternative oxidase-mediated, cyanide-resistant respiration is related to anti-stress adaptation of plants. Alternative oxidase plays a role in removal of reactive oxygen species (ROS), apoptosis inhibition, stability of plant growth rate, and increasing the resilience of plant functions (Lei *et al.*, 2008; McDonald, 2008; Watanabe *et al.*, 2008). The photosynthesis, respiration, and other physiological characteristics are also changed well in infected plants. However, the general physiological changes, especially the cyanide-resistant respiration alternation in DGIs, are less investigated.

In the present study, membrane damages, antioxidant enzyme activities, ROS levels, and chlorophyll fluorescence parameters in DGIs and yellow leaf tissues were measured. In addition, the respiratory parameters were also determined, with special attention to the cyanide-resistant respiration. We attempt to clarify the biochemical

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mechanism of formation of DGIs in *Cucumber mosaic virus* (CMV)-infected *Nicotiana tabacum* leaves.

Material and Methods

Plant growth and stress treatments

Nicotiana tabacum was grown in a temperature-controlled growth chamber with a 16 h light/8 h dark cycle at 20–25 °C. The virus isolate CMV-AH was presented by Cheng-Liang Zhang (Ministry of Agriculture Plant Quarantine Institute, China). Stock inocula of CMV were prepared and inoculated according to Xi *et al.* (2007). Approx. 15 d after virus inoculation, the mosaic phenomenon was noticeable in *Nicotiana tabacum* leaves.

Measurement of chlorophyll content

The contents of chlorophyll (Chl) *a* and *b* were determined according to Lichtenthaler and Wellburn (1983).

Determination of leaf respiration

The respiration rate was measured according to Vanlerberghe *et al.* (2002). Leaves were placed in a Clark-type oxygen electrode cuvette (Hansatech, King's Lynn, UK) at 25 °C. Inhibitors of the cytochrome pathway (1 mM KCN) and the alternative pathway (20 μM *n*-propyl gallate) were used. The alternative pathway capacity is defined as O_2 uptake rate in the presence of KCN that was sensitive to *n*-propyl gallate. The total respiration is defined as O_2 uptake rate by cucumber leaves without any inhibitor (Lei *et al*., 2008).

Oxidative damage estimation

The H_2O_2 content of leaves was measured as described by Velikova *et al.* (2000). Approx. 0.5 g of fresh leaves were cut into small pieces and homogenized in an ice bath with 5 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 20 min at 4 °C. 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance of the supernatant was read at 390 nm.

Lipid peroxidation was estimated by measuring the thiobarbituric acid-reactive substances (TBARS) as previously described (Xi *et al.,* 2007). The lipid peroxides were expressed as TBARS content.

Electrolyte leakage was measured according to Cao *et al.* (2009). After measuring the conductivity, the tobacco leaves samples were boiled for 15 min to achieve 100% electrolyte leakage.

Determination of antioxidant enzymes

The activities of superoxide dismutase (SOD) and catalase (CAT) were estimated according to Shi *et al.* (2006). The activity of peroxidase (POD) was analyzed according to the method of van Rossum *et al.* (1997).

Superoxide and H_2O_2 staining

In situ superoxide and H_2O_2 were detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB), respectively, as described previously (Yang *et al.,* 2004). Tobacco leaves were excised at the base with a razor blade and supplied through the cut ends with NBT (1 mg mL^{-1}) or DAB (0.5 mg mL^{-1}) solutions for 8 h. Leaves were then decolourized in boiling ethanol (95%) for 15 min.

Measurement of Chl fluorescence parameters in leaves

After 10 min of dark adaptation, the Chl fluorescence parameters of the leaves were measured using an FMS2 fluorescence meter (PAM-2100, Walz, Germany) according to the methods of Yuan *et al.* (2007). The following parameters were calculated using the following equations: the maximal photochemistry of PSII $Fv/Fm = (Fm - Fo)/Fm$, where Fo is the minimum fluorescence; the changes in the apparent PSII quantum yield $\Phi_{PSII} = (Fm' - Fs)/Fm'$, where Fm' is the maximum fluorescence yield after light adaptation; and the non-photochemical quenching $NPQ = Fm/Fm' - 1$.

Photosynthetic gas exchange

Photosynthetic gas exchange was measured using an open system (TPS-1, PP system, UK) according to the procedure of Yuan *et al.* (2007), using the third completely expanded leaf from the top of each plant. Leaf net photosynthetic rate (Pn) and stomatal conductance (Gs) were determined at a temperature of 25 °C, CO_2 concentration of 350 μmol mol^{-1}, 45% relative humidity, and photon flux density of 800 μmol m^{-1} s^{-1}. The leaf temperature was controlled using a leaf cuvette with an 1010-M system (TPS-1, PP system).

Statistical analysis

Means of 3 triplicates were measured. Student's *t* test was used for comparison between different treatments. A difference was considered to be statistically significant when $p < 0.05$.

Results

Chl content changes

Photosynthetic pigments are the basis of photosynthesis. The chlorophyll content of DGIs was significantly higher than of yellow leaf tissues (Fig. 1). The chloroplasts destroyed by virus infection may be resulted in the disruption of chlorophyll synthesis.

Degrees of membrane injury in different regions

TBARS are one of the most important lipid peroxidation products. The TBARS content reflects the degree of membrane injury, and is an important injury indicator of plants under stress. The TBARS content of yellow leaf tissue was significantly higher than that of DGIs and healthy tissue (Fig. 2d). H_2O_2 levels and electrolyte leakage data reflected a similar trend that the contents in yellow leaf tissues were much higher, while levels of DGIs were close to those of healthy tissues (Figs. 2b, f). These results suggest that virus infection reduces the stability and integrity of the plasma membrane, but the DGIs are less affected.

Changes of antioxidant enzymes activity

H_2O_2 is removed primarily by the enzyme CAT. The CAT activity of yellow leaf tissue was higher than those of DGIs and healthy tissue (Fig. 2a), which is consistent with the hydrogen peroxide contents. The activities of POD (which also removes H_2O_2) and SOD (which converses superoxide into H_2O_2) reflected a similar trend (Figs. 2c, e). It could be inferred that after virus infection, the enzymes activities were enhanced by ROS accumulation, which is highest in yellow leaf tissues.

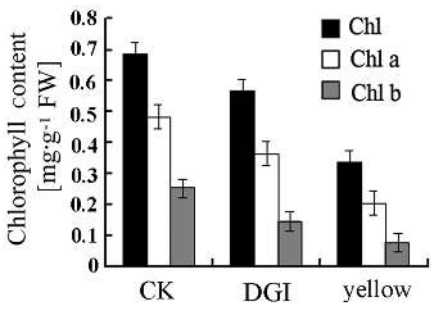

Fig. 1. Chlorophyll contents of dark green island (DGI) and yellow leaf tissue (yellow) of CMV-infected *Nicotiana tabacum* leaves. Bars represent standard deviations of 3 independent replicates ($n = 3$). CK, control.

DAB and NBT staining

DAB staining confirmed the result that the highest H_2O_2 accumulation occurred in yellow leaf tissue (Fig. 3). Contrastingly, accumulation of superoxide (stained by NBT) in the DGIs was much higher than in the yellow leaf tissue (Fig. 3).

Cyanide-resistant respiration changes

Fig. 4 shows significant changes in respiratory parameters after virus infection. The cyanide-resistant respiration intensity of yellow leaf tissue was significantly higher than that of DGIs and healthy tissue. After virus infection, the flow of glycolysis is serious disrupted; the way of pentose phosphate is still open, even strengthened. And the cyanide-resistant respiration (alternative pathway, AP) is enhanced subsequently (Rizhsky *et al.*, 2002). DGIs are the recover tissue after infection. Therefore, their physiological characteristics should be close to those of healthy tissue, although a little bit higher than those of healthy tissue.

Photosynthetic fluorescence parameters

Photosynthetic rate and stomatal conductance of yellow leaf tissue were significantly lower than those of healthy tissue, while those of DGIs were closer to healthy tissue. *F*v'/*F*m', *F*v/*F*m and Φ_{PSII} showed a similar trend. NPQ increased after infection, but not significantly in DGIs (Fig. 5). The increasing of NPQ also suggests the oxidative damages in chloroplasts (Yuan *et al.*, 2007; Liu *et al.*, 2009).

Discussion

DGIs, small pockets of uninfected, virus-resistant cells in leaves of otherwise totally infected plants, have been an enigma since they were first documented (Allard, 1914). The DGIs restrict not only RNAs but also proteins of virus. Therefore,

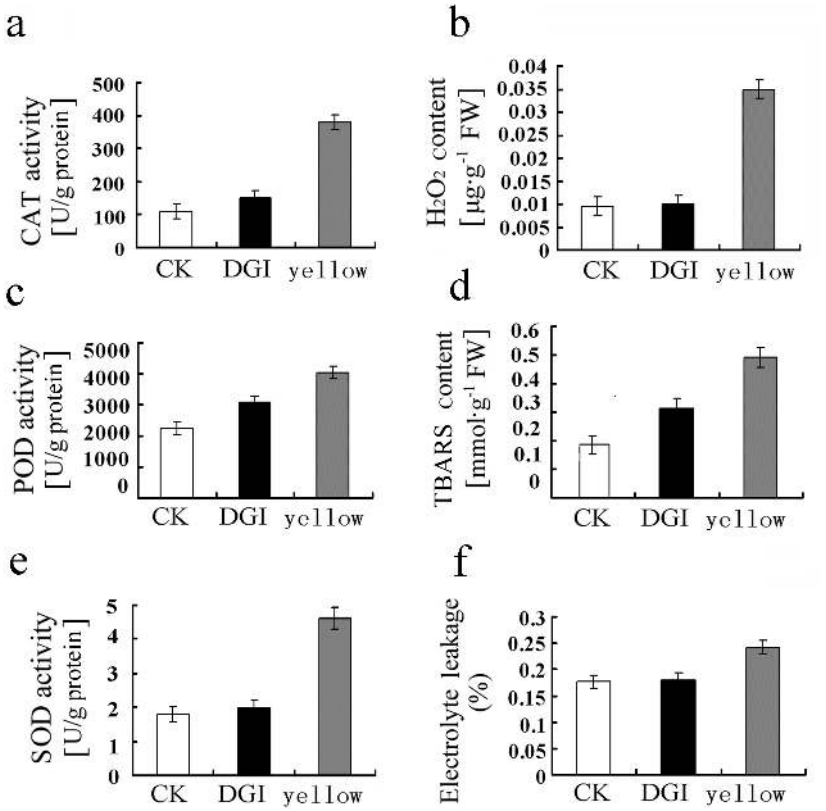

Fig. 2. Antioxidant enzyme activities, hydrogen peroxide, TBARS contents, and electrolyte leakage of dark green island (DGI) and yellow leaf tissue (yellow) of CMV-infected *Nicotiana tabacum* leaves. Bars represent standard deviations of 3 independent replicates (n = 3). CK, control.

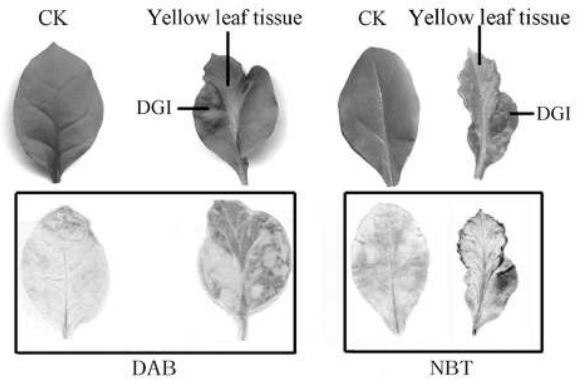

Fig. 3. DAB and NBT staining of dark green island (DGI) and yellow leaf tissue of CMV-infected *Nicotiana tabacum* leaves.

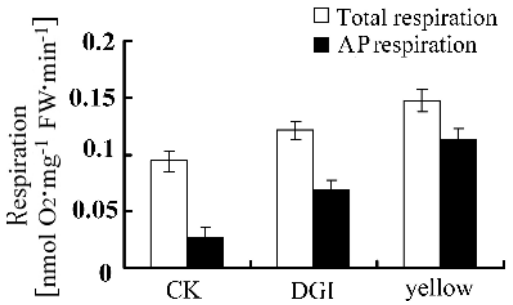

Fig. 4. Alternative pathway (AP) capacity and total respiration content of dark green island (DGI) and yellow leaf tissue (yellow) of CMV-infected *Nicotiana tabacum* leaves. Bars represent standard deviations of 3 independent replicates (n = 3). CK, control.

the DGIs can resist the secondary infection of the same virus. A systemic signal is generated by virus infection and spread throughout the plant via the phloem (Fagard *et al.*, 2000). DGIs may be initiated in dividing cells in which the signal arrives before the virus.

Our work here proved that the physical characteristics of DGIs are similar to those of healthy tissue although there are still some differences. In yellow leaf tissue, the cell structure is destructed by virus infection. The extent of membrane injury was shown by TBARS and electrolyte leakage (Nanjo *et al.*, 1999), chloroplast degradation, resulting in the blockade of pigment-protein synthesis (Moore *et al.*, 2001), and consequently reduction of chlorophylls.

An interesting phenomenon should be noted. In the early stage of infection, whole leaves yellow, and leaf edges are curled. Followed by that, some parts of the base of the leaves turn to green. ROS are considered to be a resistance signal related to this symptom (Hernandez *et al.,* 2006). The ROS level of yellow leaf tissue is much higher than that of DGIs. We infer that the generation of ROS may occur before the formation of DGIs, because DGI formation is a time-consuming process.

Another interesting phenomenon is that the distributions of superoxide and of H_2O_2 were exactly opposite after CMV infection. The content of hydrogen peroxide was closely related to the virus infection. However, superoxide was higher in DGIs than in yellow leaf tissues. This may be

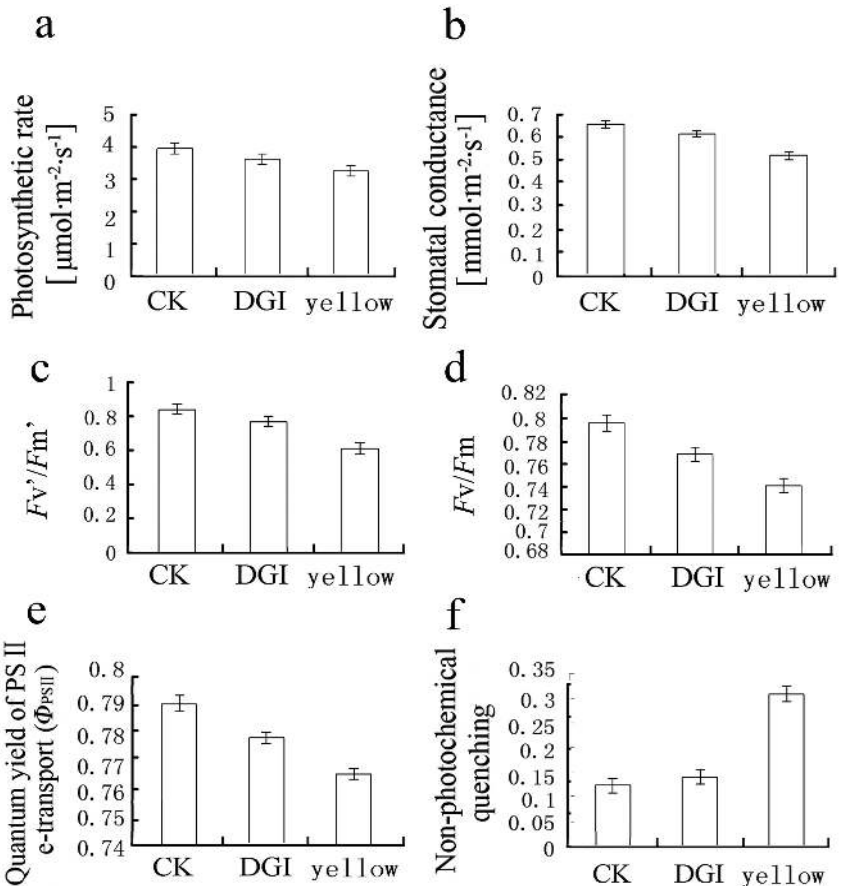

Fig. 5. Leaf net photosynthetic rate, stomatal conductance, Chl fluorescence parameters *F*v'/*F*m', *F*v/*F*m, PSII quantum yield (Φ_{PSII}), and non-photochemical quenching (NPQ) of dark green island (DGI) and yellow leaf tissue (yellow) of CMV-infected *Nicotiana tabacum* leaves. Bars represent standard deviations of 3 independent replicates (n = 3). CK, control.

due to the relatively low activity of SOD in DGIs after CMV infection; therefore superoxide generated at photosystem I under light could not be changed into hydrogen peroxide effectively.

There is a series of physiological and biochemical reactions during defense responses mediated by resistance genes. DGIs reflect post-injury recovery ability of plants. DGI is a good model to study systemic acquired resistance of plants. Further research on physiological processes and signal transductions will certainly contribute to understanding of molecular mechanisms of plant resistance to virus infection.

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Allard H. A. (1914), A review of the investigation of the mosaic disease of tobacco, together with a bibliography of the more important contributions. Bull. Torrey Bot. Club **41**, 435–458.

Atkinson P. H. and Matthews R. E. F. (1970), On the origin of dark green tissue in tobacco leaves infected with tobacco mosaic virus. Virology **40**, 344–356.

Cao Y., Zhang Z. W., Xue L. W., Du J. B., Shang J., Xu F., Yuan S., and Lin H. H. (2009), Lack of salicylic acid in *Arabidopsis* protects plants against moderate salt stress. Z. Naturforsch. **64c**, 231–238.

Fagard M., Boutet S., Morel J. B., Bellini C., and Vaucheret H. (2000), AGO1, QDE-2, and RDE-1 are related proteins required for posttranscriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. USA **97**, 11650–11654.

Fulton R. W. (1951), Super-infection by strains of tobacco mosaic virus. Phytopathology **41**, 579–592.

Hernandez J. A., Diaz-Vivancos P., Rubio M., Olmos E., Ros-Barcelo A., and Martinez-Gomez P. (2006), Long-term plum pox virus infection produces an oxidative stress in a susceptible apricot, *Prunus armeniaca*, cultivar but not in a resistant cultivar. Physiol. Plant. **126**, 140–152.

Lei T., Yan Y. C., Xi D. H., Feng H., Sun X., Zhang F., Xu W. L., Liang H. G., and Lin H. H. (2008), Effects of salicylic acid on alternative pathway respiration and alternative oxidase expression in tobacco callus. Z. Naturforsch. **63c**, 706–712.

Lichtenthaler H. K. and Wellburn A. R. (1983), Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. Biochem. Soc. Trans. **11**, 591–592.

Liu W. J., Chen Y. E., Tian W. J., Du J. B., Zhang Z. W., Xu F., Zhang F., Yuan S., and Lin H. H. (2009), Dephosphorylation of photosystem II proteins and phosphorylation of CP29 in barley photosynthetic membranes as a response to water stress. Biochim. Biophys. Acta **1787**, 1238–1245.

McDonald A. E. (2008), Alternative oxidase: an interkingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. Funct. Plant Biol. **35**, 535–552.

Moore C. J., Sutherland P. W., Forster R. L. S., Gardner R. C., and MacDiarmid R. M. (2001), Dark green islands in plant virus infection are the result of post-transcriptional gene silencing. Mol. Plant-Microbe Interact. **14**, 939–946.

Nanjo T., Kobayashi M., Yoshiba Y., Kakubari Y., Yamaguchi-Shinozaki K., and Shinozaki K. (1999), Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett. **461**, 205–210.

Rizhsky L., Liang H., and Mittler R. (2002), The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. **130**, 1143–1151.

Shi Q. H., Bao Z. Y., Zhu Z. J., Ying Q. S., and Qian Q. Q. (2006), Effects of different treatments of salicylic acid on heat tolerance, chlorophyll fluorescence, and antioxidant enzyme activity in seedlings of *Cucumis sativa* L. Plant Growth Regul. **48**, 127–135.

van Rossum M. W. P. C., Alberda M., and van der Plas L. H. W. (1997), Role of oxidative damage in tulip bulb scale micropropagation. Plant Sci. **130**, 207–216.

Vanlerberghe G. C., Robson C. A., and Yip J. Y. H. (2002), Induction of mitochondrial alternative oxidase in response to a cell signal pathway down-regulating the cytochrome pathway prevents programmed cell death. Plant Physiol. **129**, 1829–1842.

Velikova V., Yordanov I., and Edreva A. (2000), Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Protective role of exogenous polyamines. Plant Sci. **151**, 59–66.

Watanabe C. K., Hachiya T., Terashima I., and Noguchi K. (2008), The lack of alternative oxidase at low temperature leads to a disruption of the balance in carbon and nitrogen metabolism, and to an up-regulation of antioxidant defense systems in *Arabidopsis thaliana* leaves. Plant Cell Environ. **31**, 1190–1202.

Xi D. H., Feng H., Lan L.Q., Du J. B., Wang J. H., Zhang Z. W., Xue L. W., Xu W., and Lin H. H. (2007), Characterization of synergy between cucumber mosaic virus and tobacco necrosis virus in *Nicotiana benthamiana*. J. Phytopathol. **155**, 570–573.

Yang Y. N., Qi M., and Mei C. S. (2004), Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. Plant J. **40**, 909–919.

Yuan S., Liu Z. L., Liu W. J., Lei T., Luo M. H., Du J. B., Wang J. H., and Lin H. H. (2007), A chlorophyll-less barley mutant "*NYB*" is insensitive to water stress. Z. Naturforsch. **62c**, 403–409.