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ARTICLE

F690-F740 is more suitable than F690/F740 for mapping the regeneration of Cd-induced chlorosis in poplar leaves by fluorescence imaging

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ABSTRACT Pattern of chlorophyll distribution cannot be typified by measuring chlorophyll content of samples collected throughout the leaf. Chlorophyll fluorescence imaging is a widely used method for mapping these changes. Cd caused strong chlorosis, which could be regenerated by the addition of surplus iron. Regeneration in the presence or absence of Cd resulted in different leaf phenotypes with mosaic chlorophyll patterns. The parameters used for imaging chlorophyll distribution described in the literature (such as F690/F740) proved to be insufficiently sensitive to map the small-scale changes observed during regeneration of the chlorotic leaves of Cd treated plants. Imaging F690/F740 only gave a picture with little contrast in relation to spatial changes. In contrast, a new parameter, F690-F740 difference, was suitable for correctly imaging the changes in chlorophyll distribution during the surplus iron induced greening process..
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KEY WORDS

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Cd has been shown to strongly influence the development of the photosynthetic apparatus causing premature senescence of chloroplasts (Krupa and Baszyński 1995; Mishliwa-Kurdziel et al. 2002). Cd inhibited chlorophyll (Chl) synthesis (Padmaja et al. 1990; Mishliwa-Kurdziel and Strzalka 2002a) and the stable binding of Chls to proteins (Horváth et al. 1996) thereby decreasing the accumulation of pigment-lipoprotein complexes, particularly PSI (Sárvári 2005). However, Cd effects on the photosynthetic apparatus, including chlorosis, could be recovered by the addition of surplus iron in a week-time (Solti et al. 2007).

Fluorescence is a useful tool to characterize several plant stresses. Multicolour fluorescence imaging is an important, non-invasive technique to collect spatially detailed information on leaf physiology. Fluorescence emission filtered at the main peaks of the leaf fluorescence emission spectra after exciting the specimen with UV flashes and obtained from different points of the leaf surface gives the possibility of imaging the distribution of fluorescent components including Chls (Daley et al. 1989; Buschmann et al. 2000). At room temperature, the main origin of Chl fluorescence is the photosystem (PS) II emitting around 690 nm. However, there are several studies showing that this fluorescence is modified by the emission of PSI around 740 nm (Agati et al. 2000; Buschmann 2007). Variations in the red/far red ratios such as F690/F740 negatively correlate with changes in Chl content, thus after calibration, changes in the amount and distribution of Chl in leaves can be followed in vivo (Hák et al. 1990; Lichtenthaler et al. 1990; D'Ambrosio et al. 1992; Buschmann 2007). Many types of stresses causing chlorosis can be easily detected by this parameter (Balachandran et al. 1994). However, a more sensitive parameter may be needed in the case of mapping small-scale changes such as regeneration of Cd-induced chlorosis.

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Materials and Methods

Experiments were performed on poplar (Populus glauca Haines, 1906 var. Kopeczkii) plants, grown and treated in hydroponics (Hoagland solution of ¼ strength, iron source 10 µM Fe-citrate), in growth chamber with 12/12 hours light (100 µE m^{-2} s^{-1})/dark periods, 20/18°C and 70/75% relative humidity. Plants were treated with 10 µM $Cd(NO_3)_2$ from their four-leaf stage and regenerated after one week of treatment by the addition of 50 µM Fe-citrate in the presence or absence of 10 µM $Cd(NO_3)_2$.

Chl contents were determined according to Porra et al. (1989) and carotenoid contents were determined as in Tóth et al. (2002), respectively.

A compact flash-lamp fluorescence imaging system was used for imaging the fluorescence at 690 nm and 740 nm (Lichtenthaler and Babani 2000). Excitation was carried out

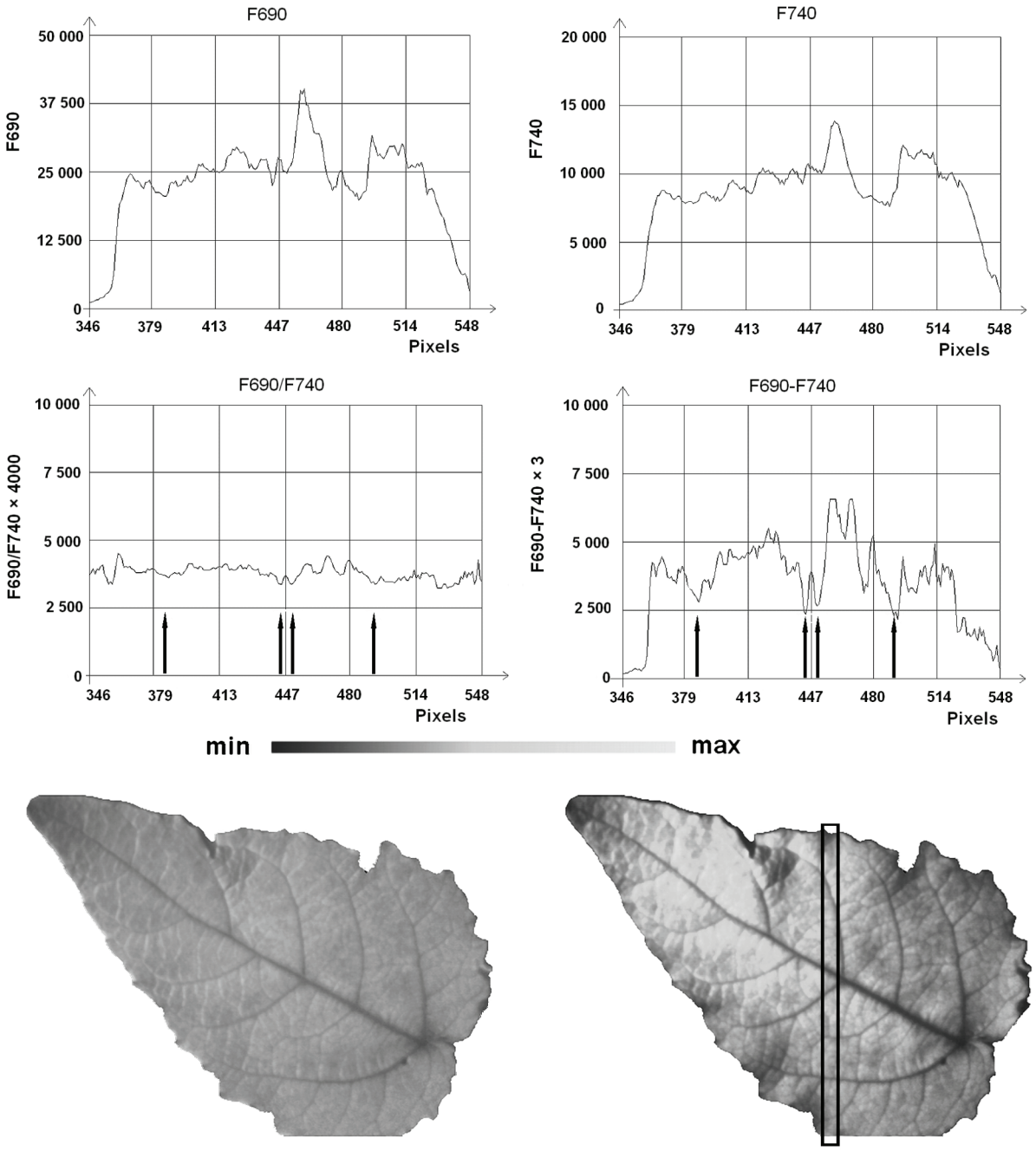

Figure 1. Top: Vertical profiles of the distribution of the 690 nm and 740 nm fluorescence within the leaf of a plant regenerated by 50 μM iron in the absence of Cd for one day. Middle: Vertical profiles of the distribution of F690/F740 and F690-F740 parameters of the same leaf. Arrows show the sites of higher Chl content next to the great leaf veins. Bottom: distribution of F690/F740 (left) and F690-F740 (right) parameters on the leaf area. Black bracket on F690-F740 image shows the section of the leaf where the vertical profiles were taken.

by xenon-lamp flashes of 16.7 Hz filtered by a DUG 11 filter (Schott, Mainz, Germany) to ensure the appropriate exciting wavelength (λ_{exc}=360–370 nm). The detection of fluorescence was performed by CCD video camera (objective: Nikon-AF Nikkor, Japan, 1:1.4 D, 50 mm Ø) at the above-mentioned wavelengths using appropriate interference filters. Accumulation of 400 images was chosen as a suitable number of successive readout images. Images were taken from the adaxial side of light-adapted leaves at room temperature and corrected by the filter sensitivity parameters and the inhomogeneity of

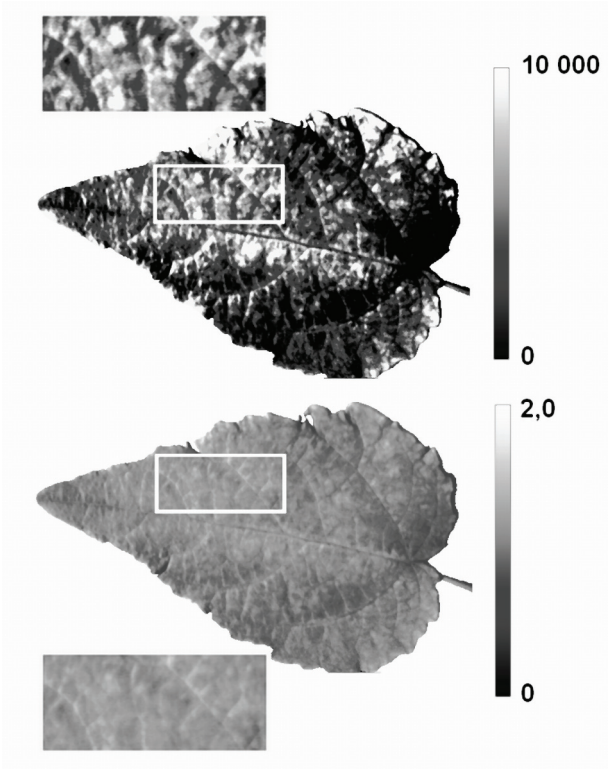

Figure 2. Image of F690-F740 difference (top) and F690/F740 ratio (bottom) of a leaf regenerated by 50 μM iron in the presence of 10 μM Cd for one week. The parts of the pictures in the brackets are magnified outside the images for a better comparison of the distributional inhomogeneity. Lighter points on the leaf area, which are typical of this kind of regeneration, are well shown by the F690-F740 fluorescence difference.

the exciting light. Corrections and arithmetical operations were performed by Camille 1.05 software (Photonetics, Kehl, Germany).

Results

Cd induced decrease in Chl content and the increased carotenod/Chl ratio, particularly in violaxanthin cycle carotenoids, could be regenerated using surplus iron applied at 50 μM concentration both in the presence and the absence of Cd (Table 1). The increase of Chl content was found to correlate with the decrease of both F690/F740 and F690-F740 parameters. This re-greening, which altogether lasted for a week, began next to the leaf veins and in the basal part of the leaf (Fig. 1 bottom). This process is well shown by the vertical profile of F690-F740 difference image of the leaf fluorescence but can be hardly seen in the profile of F690/F740 ratio due to the ground noise coming from the light reflexion of leaves (Fig. 1 middle). The F690-F740 difference, gave a better resolution due to the better contrast.

Table 1. Changes in the Chl content, carotenoid/Chl ratio, F690/F740 and F690-F740 difference under regeneration. Plants had developed Cd symptoms at the 0. day of recovery. Regenerated type 1 and type 2 mean recovery in the absence and presence of Cd, respectively.

Recovery	control	regenerated	
		type 1	type 2
		μg Chl cm^{-2} leaf area	
0. day	15,6±3,4	11,0±0,5	
2. day	18,7±0,6	13,1±2,6	12,1±1,9
7. day	25,5±1,6	22,8±1,0	16,9±2,4
		carotenoid/Chl ratio	
0. day	0,150±0,003	0,183±0,001	
2. day	0,148±0,002	0,173±0,004	0,177±0,009
7. day	0,142±0,004	0,151±0,006	0,157±0,001
		F690/F740	
0. day	0,625±0,004	1,657±0,057	
2. day	0,630±0,013	0,947±0,025	1,028±0,008
7. day	0,551±0,002	0,651±0,013	0,967±0,046
		F690-F740	
0. day	-5592,4±937,7	8278,1±276,1	
2. day	-6863,1±962,4	-751,0±361,3	1171,0±371,3
7. day	-10086,3±372,0	-6165,0±230,4	421,1±146,7

Leaves of plants regenerated with surplus iron in the presence of Cd seemed to show a macroscopic mosaic pattern of regions with higher or lower Chl content, though their mean Chl content was similar to that of control leaves. It means stimulation or a lower level of recovery in a small scale (Fig. 2, top). This mosaic pattern, which is well observable on the F690-F740 difference image, is undetectable by imaging the F690/F740 ratio (Fig. 2, bottom).

Discussion

Chl fluorescence imaging means a useful technique to determine the leaf Chl content and to follow its changes in concentration and pattern (Buschmann 2007). Such differences could not be determined in organic solvents by collecting samples from the different part of the leaf. The well-known red/far red ratios are appropriate to measure Chl content due to the linear correlation of the two parameters (Lichtenthaler et al. 1990). However, they have less sensitivity to follow small changes, such as the increase in Chl content in regenerating chlorotic leaves (Hák et al. 1990) or in the case of the recovery of Cd induced chlorosis, where it was a sharp increase in the Chl content along the great leaf veins (Fig. 1) or the leaves developed a mosaic picture during re-greening (Fig. 2). To map fine-pattern changes, F690/F740 ratio is less useful because the difference in the numeric values of leaf regions with different Chl contents are too small, and even making images by multiplied values, the differences between these points are not enhanced sufficiently to study distributional

patterns. Ratios are not sensitive to the amplitude of the fluorescent signal, so the ratio coming from the background may be similar to that of the leaf. The resolution can be improved by subtracting the background values. However, it means a couple of arithmetical work, which makes it unsuitable to use in routine experiments.

For imaging at low Chl concentrations, the value of F690-F740 difference is much more appropriate. It does not change the distribution but strengthens the small-grained pattern by giving larger pixel differences (Fig. 1). Its value can be used for making images without any other arithmetical operations, so in case of evaluating many samples it proved to be more useful than F690/F740. The fluorescence difference shows higher sensitivity to lower Chl concentrations compared to F690/F740 ratio because the fluorescence at 690 nm is considerably higher at lower Chl content due to the lower level of re-absorption of emission. Thus, the correlation of the F690-F740 parameter and the Chl content is rather exponential than linear, and small elevation in the Chl content results in large intensity differences in the value of the parameter. In addition, both the decrease in the 690 nm fluorescence due to the increase in Chl content and the increase of 740 nm fluorescence connected with regeneration of PSI (Solti et al. 2007) contribute to the greater sensitivity of this parameter in the case of Cd treated leaves. This attribute also makes F690-F740 difference suitable to characterize the mosaic pattern of leaves in plants regenerated in the presence of cadmium (Fig. 2). However, the parameter has less sensitivity to green leaves with higher Chl content, which can be better characterized by the F690/F740 ratio.

In conclusion, F690-F740 difference gives a better view of the leaf greening and small-scale inhomogeneity of Chl content than F690/F740 ratio.

Acknowledgement

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