Acta Physiol Plant (2016) 38:102
DOI 10.1007/s11738-016-2113-y

REVIEW

Chlorophyll *a* fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions

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Received: 11 April 2015 / Revised: 29 February 2016 / Accepted: 1 March 2016 / Published online: 29 March 2016

Abstract Plants living under natural conditions are exposed to many adverse factors that interfere with the photosynthetic process, leading to declines in growth, development, and yield. The recent development of Chlorophyll *a* fluorescence (ChlF) represents a potentially valuable new approach to study the photochemical efficiency of leaves. Specifically, the analysis of fluorescence signals provides detailed information on the status and function of Photosystem II (PSII) reaction centers, light-harvesting antenna complexes, and both the donor and acceptor sides of PSII. Here, we review the results of fast ChlF analyses of photosynthetic responses to environmental stresses, and discuss the potential scientific and practical applications of this innovative methodology. The recent availability of portable devices has significantly expanded the potential utilization of ChlF techniques, especially for the purposes of crop phenotyping and monitoring.

Keywords Chlorophyll fluorescence · JIP-test · Photosynthesis · Photosystem II · Quantum efficiency · Stress detection

Abbreviations

ABS	Absorption flux
Chl	Chlorophyll

Communicated by AK Kononowicz.

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ChlF	Chlorophyll fluorescence
CS	Cross section of the sample
Cyt b_6f	Cytochrome b_6f
DF	Delayed (chlorophyll) fluorescence
DFI	Drought factor index
LHC (II)	Light-harvesting complex (of PSII)
OEC	Oxygen-evolving complex
P680*	Excited PSII reaction center
P700	PSI reaction center
PAR	Photosynthetically active radiation
PC	Plastocyanin
PCA	Principal component analysis
PF	Prompt (chlorophyll) fluorescence
Pheo	Pheophytin
PQ	Plastoquinone
PSI, PSII	Photosystem I, II
Q_A	Primary plastoquinone electron acceptor of PSII
Q_B	Secondary plastoquinone electron acceptor
RC	Reaction center
ROS	Reactive oxygen species

Introduction

Over the course of the 21st century, global agriculture must produce more food to sustain a growing human population (Beddington et al. 2012). However, this goal is threatened by anthropogenic climate change which has the potential to dramatically reduce yields in affected regions (Lobell et al. 2008). Recent studies indicate that Chlorophyll fluorescence (ChlF) measurements may provide unique benchmarks to improve global agricultural productivity models, improving the reliability of crop yield projections under climate change scenarios (Guanter et al. 2014; Malaspina et al. 2014). More generally, ChlF is emerging as a very powerful tool in agricultural, environmental, and ecological studies (Gottardini et al. 2014). One of its main advantages is that ChlF is a non-invasive tool, allowing scientists to get information on the photosynthetic process without destroying the tested sample.

Under natural conditions, plants are exposed to many adverse environmental stress factors. These can disrupt the photosynthetic apparatus, causing a decrease of plant productivity and overall yield. Photosynthesis is particularly sensitive to environmental constraints (see Kalaji et al. 2012), making photosynthetic measurements an important component of plant stress studies. However, traditional methods, even technically advanced ones such as the measurements of photosynthetic rates through gas exchange (CO_2, H_2O, and O_2), are time-consuming and provide incomplete information on overall photosynthetic function. In contrast, ChlF measurements represent a simple, non-destructive, inexpensive and rapid tool for analyzing light-dependent photosynthetic reactions and for indirectly estimating chlorophyll content within the same sample tissue (See reviews by Govindjee 1995; Papageorgiou and Govindjee 2011; and by Stirbet and Govindjee 2011, 2012). These technical advantages of ChlF approaches have made it a popular technique among plant breeders (e.g., for crop phenotyping and monitoring), biotechnologists, plant physiologists, farmers, gardeners, foresters, ecophysiologists, and environmentalists.

Critically, from the perspective of plant stress studies, ChlF measurements also provide indirect information about the physiological condition of plants. Analysis of chlorophyll fluorescence (ChlF) induction curves allows the evaluation of the physiological condition of photosystem II (PSII) and photosynthetic electron transport chain components. It also provides information on the cooperation of light-dependent photochemical reactions and light-independent biochemical reactions. More generally, ChlF measurements relate, directly or indirectly, to all stages of light-dependent photosynthetic reactions, including photolysis of water, electron transport, pH gradient formation across the thylakoid membrane, and ATP synthesis and thus general bioenergetic condition of the photosynthetic machinery (Bernát et al. 2012).

Numerous ChlF techniques and applications have now been developed, each one contributing to knowledge of photosynthesis. In this review, we focus on results from fast fluorescence analysis induced by continuous illumination. These studies were made possible by the development of a reliable mathematical model known as the JIP-test (Strasser et al. 2004) that allowed the analysis of fluorescence changes that occur in less than 1 s. Such analyses provide detailed information on the status and function of PSII reaction centers, antenna, as well as on donor and acceptor sides of PSII. The main focus of the review is to outline the effects of stress factors on the photochemical processes as reflected in changes in fast ChlF kinetics and related biophysical parameters.

Analysis of polyphasic chlorophyll fluorescence kinetics

Illumination of a dark-adapted photosynthetic sample allows a polyphasic chlorophyll fluorescence induction curve to be obtained (O–J–I–P-transient) (Fig. 1). The curve's trajectory provides considerable information about the structure and function of the photosynthetic apparatus (Kautsky and Hirsch 1931; Schreiber et al. 1994). The JIP-test is based on the rise in polyphasic fast chlorophyll *a*,

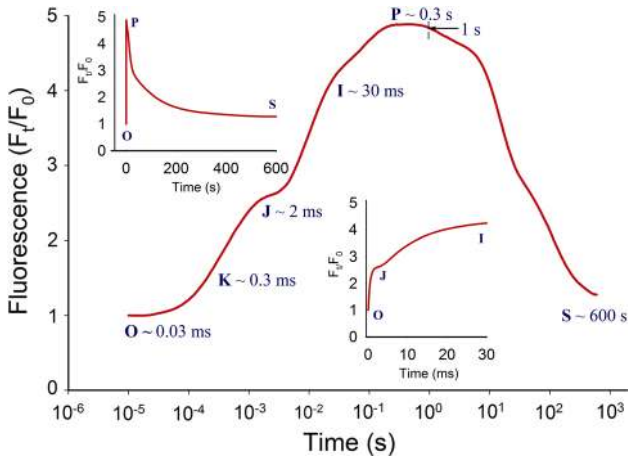

Fig. 1 A typical Chlorophyll *a* polyphasic fluorescence curve, exhibited by plants (main *plot*). The transient is plotted on a logarithmic time scale from 10 μs to 600 s. The same *curve* plotted in regular time scale is shown in upper insertion (*left*). Initial part of OJIP transient (0–30 ms) plotted on regular time scale is shown in second insertion (*bottom*). The *marks* refer to the selected time points used by the JIP-test for the calculation of structural and functional parameters. The signals are the fluorescence intensity F_o (at 30 μs); the fluorescence intensity F_K (at 300 μs); the fluorescence intensities F_J (at 2 ms) and F_I (at 30 ms); the maximal fluorescence intensity, $F_P = F_M$ (at time denoted as t_{FM}). Usually, for analysis of fluorescence transient, the record is limited to 1 s, creating typical OJIP-polyphasic fluorescence rise

and is used for investigating the correlation between light-dependent reactions and ChlF. It is based on the theory of "energy flow" across thylakoid membranes (Strasser et al. 2000). This theory can be operationalized in simple algebraic equations, representing the balance between total energy inflows and outflows for each of the examined light-harvesting complexes and providing information on the probable distribution of absorbed energy. With these equations, it is possible to describe the energetic communication (also known as the "grouping" or "connectivity" and "overall grouping probability") between the PSII complexes (Stirbet 2013).

The name of the JIP-test (OJIP) originates from the specific points on the induction curve formed by the recorded ChlF signal (Fig. 1): these correspond to the gradual reduction of Q_A and the primary electron acceptor of PSII. The shape of the curve depends from PSII grouping (L-band) (Tsimilli-Michael and Strasser 2013) and the balance between electron donation from OEC → $P680^+$ and electron accept from Q_A^- (K-band) (Strasser et al. 2005). The O–J part of the fluorescence rise relates to the closure of some of the PSII reaction centers in response to the reduction of Q_A to a level determined by the ratio between the trapping rate and Q_A reoxidation rate by Q_B and the rest of the electron transfer chain. The J–I part of the curve corresponds to the reduction of the secondary electron acceptor Q_B, plastoquinone (PQ), cytochrome (Cyt b_6f), and PC. The increase in ChlF in the I–P part of the induction curve is typically attributed to the reduction of electron transporters (ferredoxin, intermediary acceptors, and NADP) of the PSI acceptor side. Stress conditions such as high temperature, excessive PAR, nitrogen deficiency, or drought inhibit the oxygen-evolving complex (OEC) and block the electron transport between the OEC and tyrosine (Guha et al. 2013). Under stressful conditions, a peak occurs (the K-band) within the 200–300 μs range of the ChlF induction curve, indicating a disruption of the OEC.

The JIP-test parameters characterizing the PAR energy absorption and electron transport can be categorized into four main groups: (1) basic measured and calculated values [fluorescence (F_t) and variable fluorescence (V_t) values, initial slope, etc.]; (2) quantum yields and probabilities; (3) energy fluxes; and (4) vitality indices. The biophysical parameters representing the energy fluxes are divided into specific and phenomenological. The specific parameters are calculated per reaction center (RC), while the phenomenological parameters are calculated per sample cross section (CS).

The vitality indices represent the products of several independent parameters combining structural and functional criteria. These criteria include the density of reaction centers, the quantum efficiency of primary photochemistry, and conversion of excitation energy in electron transport (Strasser et al. 2000, 2004, 2010; Zushi et al. 2012). The vitality indices were created as non-specific parameters to be used mostly in practical applications, such as screening for enhanced stress tolerance underfield conditions (Srivastava et al. 1999; Strasser et al. 2004; Brestic and Zivcak 2013).

Chlorophyll fluorescence kinetics can also be used to reveal PSII heterogeneity of photosynthetic apparatus. PSII is naturally heterogeneous in terms of antenna and reducing side. Antenna heterogeneity includes variations in antenna size and in connectivity (grouping) of antenna molecules. Based on antenna size, PSII centers have been classified as alpha (α), beta (β), and gamma (γ) (Melis and Homann 1976). These differ from each other in life span and number of associated chlorophylls. Reducing side heterogeneity is related to the ability to transfer an electron from Q_A. Centers which are capable of transferring electrons from Q_A to Q_B are termed Q_B reducing centers, while those which are unable to do so are termed Q_B non-reducing centers. Specific characteristics of PSII heterogeneity have been reviewed in Jajoo (2013). Recent studies have shown changes in PSII heterogeneity under high temperature (Mathur et al. 2011b), high salt (Mehta et al. 2010a), and some pollutants like polycyclic aromatic hydrocarbons (PAH) (Tomar and Jajoo 2013, 2014). Changes may relate to the number of active/inactive reaction centers,

interconversion of active alpha centers into inactive beta and gamma centers, and increases in the number of Q_B non-reducing centers under various stress conditions.

Chlorophyll fluorescence kinetic parameters in response to various abiotic stresses

In the following sections, we review the evidence that ChlF kinetics may serve as an useful indicator of the negative impacts of climate change and human activities, such as high and low temperature, drought, salinity, nutrient deficiencies, and heavy metals.

Temperature effects

Climate change is likely to increase heat stress in plants, limiting productivity and biomass production. Photosynthesis is the most sensitive of plant cell processes to high temperatures (Sharkey and Schrader 2006), which cause changes in the reduction–oxidation properties of PSII acceptors and reduce the efficiency of photosynthetic electron transport in both photosystems (Mathur et al. 2014).

Heat stress affects the values of ChlF parameters (Fig. 2a). For example, in response to high temperature stress apples *Malus* x *domestica* Borkh reduced both the ratio of reduced acceptors (plastoquinone) Q_A^- to RC and the ratio of reduced acceptors (plastoquinone) Q_B^- to Q_A^-. There was also a decrease in the maximum quantum yield of PSII and an increase in the minimal fluorescence value (Chen et al. 2009; Brestic et al. 2013). High temperature stress also influences the shape of the O–J–I–P curve, decreasing F_M and increasing F_o. The increase in F_o may be due to the release of LHC II from the PSII complex, inactivation of PSII photochemical reaction or an inhibition of electron flow due to the reduced transfer of Q_A to Q_B (Mathur et al. 2011a). For example, the increase of F_o observed in spinach and rice has been attributed to the irreversible dissociation of LHC II from the PSII complex and partly reversible inactivation of PSII (Yamane et al. 1997). The decrease in the fluorescence F_M level may be related to denaturation of chlorophyll-proteins (Yamane et al. 1997).

The K peak (at 300 μs) is a well-documented symptom of heat stress, and is thought to indicate the separation of the OEC complex and electron transport between pheophytin and primary electron acceptor Q_A (Strasser et al. 2000; Lazár 2006). In wheat, a treatment at 35 °C had no affect on photosynthetic efficiency, while exposure to 45 °C caused irreversible damage to the OEC (Schreiber et al. 2012). The direct cause of the ChlF curve peak (K) is the outflow of electrons from P680 to PSII acceptors, which over-compensates the inflow of electrons from the donor side of PSII to P680. The K peak is also affected by changes in the energetic relationships between photosystems II. An increase in the F_K/F_J ratio (Srivastava and Strasser 1995) indicates that the heat stress is inhibiting the donation of electrons by the OEC.

The fast ChlF technique also represents a useful tool to monitor PSII thermostability. The most efficient approach is to estimate the critical temperature, i.e., the threshold level above which there is a sharp increase/decrease of the observed parameter (Brestic and Zivcak 2013). Some genotypes can serve as donors of enhanced heat tolerance in crop breeding programs. For example, the response of heat-treated common bean (*Phaseolus vulgaris* L.) lines and their recovery were monitored by changes in ChlF induction and analyzed by means of the JIP-test (Stefanov et al. 2011). PSII thermostability of 30 genotypes of Winter wheat plants (*Triticum aestivum* L.) with different geographic origins were identified using the fast ChlF kinetics (Brestic et al. 2012). ChlF has also been shown to be a more effective than conventional methods (e.g., harvest index, grain filling, etc.) for screening genotypes of durum wheat (Gautum et al. 2014).

In certain latitudes, low temperatures are a major factor limiting crop yields (Yang et al. 2009). In the northern hemisphere, low temperatures during the winter and early spring are usually followed by intense PAR. These conditions can cause degradation of the thylakoid structure and distortion in light-dependent photosynthetic reactions (Suzuki et al. 2011). Cold stress also affects ChlF parameters (Fig. 2b). For example, a decrease was observed in chlorophyll content, OEC efficiency on the donor side of PSII, photochemical quenching, and efficiency of open PSII reaction centers for bitter melon plants (*Momordica charantia* L.) exposed to cold stress (Yang et al. 2009). Some plant species are known for their tolerance to low temperatures, showing less photoinhibition of PSII. For example, under cold stress pea plants show only small modifications in ChlF parameters (Strauss et al. 2006; Streb et al. 2008).

Drought stress

Drought stress effects on photosynthetic apparatus are well known. They typically start with mostly stomatal effects at moderate drought intensity, and culminate in metabolic and structural changes caused by severe or long-lasting drought stress (Jedmowski et al. 2013). This final changes are also associated with enhancement of photoprotective and antioxidant functions and pathways (Chaves et al. 2009). PSII has high resistance to water deficit (compared to PSI) and negative impacts therefore only occur under conditions of extreme drought (Lauriano et al. 2006).

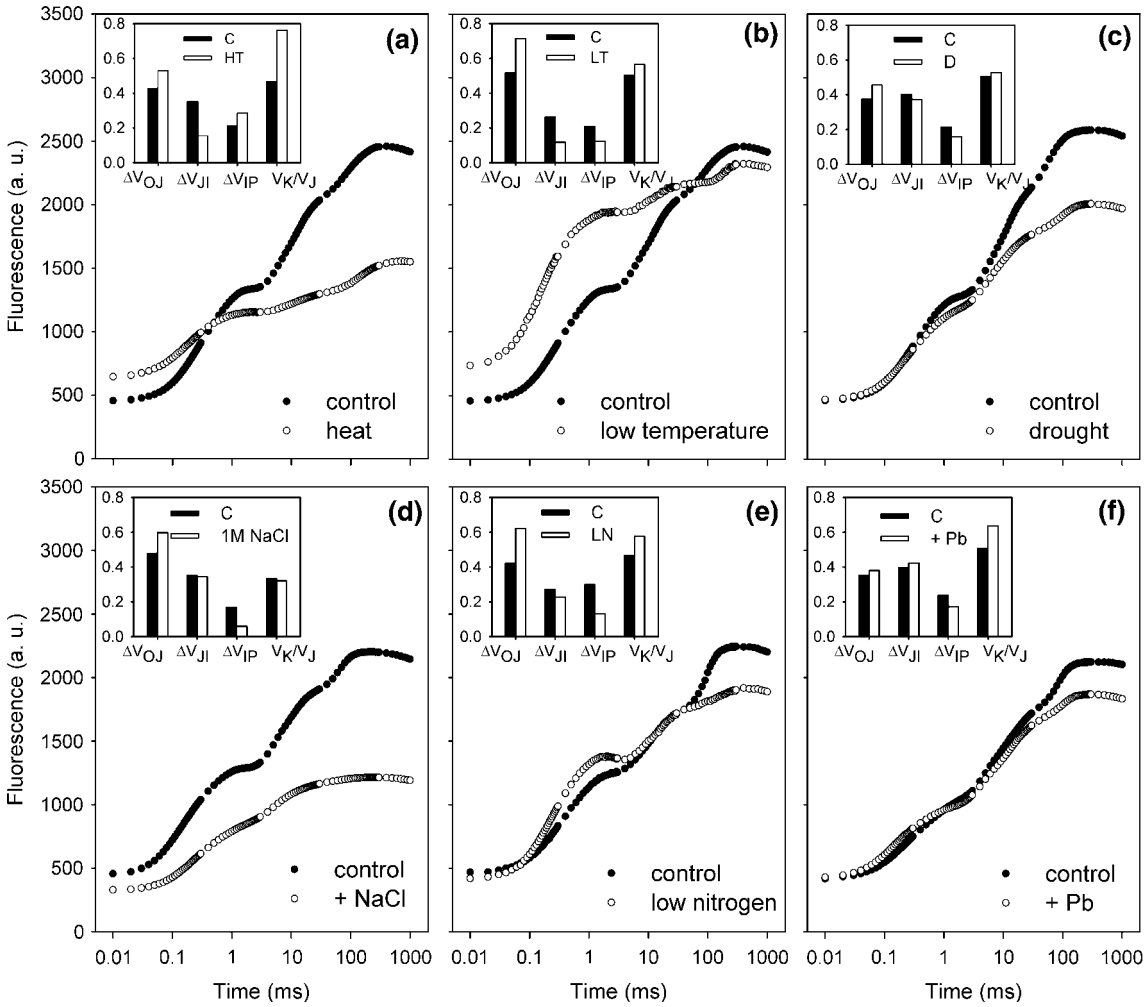

Fig. 2 The O(K)JIP-transients of chlorophyll fluorescence in wheat (*Triticum* sp. L.) plant samples exposed to different stress conditions compared to non-stressed plants. The insertions show the changes of amplitude of relative variable fluorescence in O–J phase (ΔV_{OJ}), J–I phase (ΔV_{JI}), I–P phase (ΔV_{IP}) and of the ratio of variable fluorescence in time 0.3 ms (V_K/V_J) to variable fluorescence in time 2 ms (V_J) as an indicator of the PSII donor side limitation (K-band). Individual *graphs* present comparisons of records in non-stressed plants (control, C) of **a** exposed to heat stress (8 h exposed to high temperature in moderate actinic light, the leaf temperature was ~40 °C); **b** exposed to long-lasting suboptimal low temperature (10 days at 10/6 °C day/night); **c** exposed to severe drought stress (12th day after withholding of irrigation, leaf relative water content ~60 %); **d** exposed to salt (NaCl) stress; **e** plants cultivated at low soil nitrogen content (low nitrogen, LN); and **f** exposed to lead (Pb). Data were provided by the authors of this review

ChlF measurements indicate enhanced protection of PSII and PSI photochemistry under drought conditions by adjusting the energy distribution between photosystems and by activating alternative electron sinks (Zivcak et al. 2013). Drought stress may enhance the resistance of PSII to heat stress as shown by the disappearance of the K-band from the OJIP transient (see Fig. 2c and Oukarroum et al. 2012).

The ChlF method is potentially useful for screening genotypes for drought tolerance (Guha et al. 2013). The fluorescence rise during the first 2–3 ms is related to primary photochemistry and it has been suggested that stimulated L- and K-bands can be used as tools for evaluating potential to cope with (and recover from) drought stress (Oukarroum et al. 2007). The L-band is influenced by the excitation energy transfer between PSII units, commonly denoted as connectivity or grouping (Strasser and Stirbet 1998).This can be influenced composition of PSII antennae that have been changed due to mutations (Brestic et al. 2014) or environmental conditions (Zivcak et al. 2014a). The K-band has been associated with a dissociation of the oxygen-evolving complex (OEC) (Guissé et al. 1995). The measurement of OLKJIP fluorescence transients and their analysis using the JIP-test might therefore be used as indicators for drought stress tolerance and physiological disturbances before the appearance of visible signs of drought stress.

The most widely used parameter from the ChlF OJIP transient is the performance index (PI), which provides

quantitative information about the general state of plants and their vitality. PI is the product of three independent characteristics: the concentration of reaction centers per chlorophyll, a parameter related to primary photochemistry and a parameter related to electron transport (Strasser et al. 2004). PI is therefore sensitive to changes in either antenna properties, trapping efficiency or electron transport beyond Q_A. For example, the PI of winter wheat decreased during prolonged post-anthesis drought stress. Moreover, the drought tolerance of wheat genotypes estimated from PI values recorded in drought stress also correlated well with the drought tolerance assessed by grain yield (Zivcak et al. 2008). PI is closely related to the drought factor index (DFI), which represents the relative drought-induced reduction of PI during a freely defined time of drought stress. A DFI approach was used by Strauss et al. (2006) to detect dark chilling tolerance in soybean genotypes. DFI has also been used to rank drought stress tolerance in 10 barley varieties (Oukarroum et al. 2007) and 21 mutant germplasms of sesame (Boureima et al. 2012). The most tolerant and the most sensitive races of barley and *Sorghum bicolor* from Egypt were identified using the PI parameter and the ChlF fast induction curve (Jedmowski et al. 2013). These studies demonstrate that drought-tolerant and drought-sensitive cultivars can be differentiated at the level of PSII. An increase of ABS/RC ratio under drought stress has also been observed (Van Heerden et al. 2007; Gomes et al. 2012), possibly due to inactivation of some PSII RCs or an increase in antenna size.

Drought stress can also affect the relative amplitude of the I–P phase from OJIP curve. The I–P phase has been recorded as the slowest phase of the fluorescence rise (approximately 30–200 ms) and was parallel the re-reduction of plastocyanin PC^+ and $P_{700}{}^+$ in PSI (Schreiber et al. 1989; Schansker et al. 2003). The I–P phase seems to be related to the content of PSI reaction centers (Ceppi et al. 2012) or availability of linear electron transport as determined by 820-nm transmission measurements (Zivcak et al. 2014a). For example, the extent of the I–P loss in barley varieties depends on their drought tolerance (Oukarroum et al. 2009; Ceppi et al. 2012). ChlF is emitted following a dark-to-light transition of a photosynthetic sample, while delayed fluorescence emission (DF) occurs during light-to-dark transitions (Goltsev et al. 2009; Strasser et al. 2010; Kalaji et al. 2012). DF is thought to reflect the recombination (in the dark) between the reduced primary electron acceptor Q_A^- and the oxidized donor ($P680^+$) of PSII that are formed after light-induced charge separation. The shape of the DF induction curve depends on the sample type and its physiological state. Simultaneous measurements of Chl *a* fluorescence and DF have recently been developed to obtain rate constants for different photosynthetic reactions (Strasser et al. 2010). Using this technique, Goltsev et al. (2012) observed that reoxidation of Q_A^- was inhibited during drought stress and that quantum yields of photoinduced electron transport in PSII reaction center to Q_A were suppressed and that the fast phase of photoinduced kinetics of the modulated reflection signal was reduced.

Salinity stress

Plant responses to salinity stress are determined by many aspects, such as the expression of specific genes, plant development stage, and glycine betaine accumulation which protects the photosynthetic apparatus by stabilizing the external proteins of the PSII complex (Murata et al. 1992). Salinity stress disrupts the electron transport from the RCs to the plastoquinone pool (Strasser et al. 2000; and Fig. 2d). Schreiber et al. (1994) identified the OEC as one of the most sensitive components in the photosynthetic electron transport chain. Its reduced performance is usually caused by an electron transport disorder. Modifications can also be observed in ChlF parameters and PSII functioning. Under high salinity conditions, electron trapping in PSII reaction center becomes less efficient due to the dissociation of LHCII and PSII (Havaux 1993). A decrease in maximum quantum yield of PSII and an increase in non-photochemical quenching have been recorded in a number of species, including barley (Kalaji and Rutkowska 2004), cultivated tobacco *Nicotiana tobacum* L. (Yang et al. 2008), and even among certain halophytes, such as *Sarcocornia fruticosa* L. Moreover, in tomatoes and cucumber *Cucumis sativus* L. seedlings the following parameters were reduced during salinity stress: PSII efficiency in light, electron transport chain efficiency, and the efficiency of PSII open reaction centers in light (He et al. 2009; Zhang and Sharkey 2009). The damage caused by salinity stress in wheat was more prominent at the donor side rather than the acceptor side of PSII, and this damage was fully reversible ($\sim$100 %) at the acceptor side of PSII, while recovery of the donor side was less than 85 % (Mehta et al. 2010b). The osmotic and ionic effects of salinity stress have also been differentiated using ChlF measurements (Singh-Tomar et al. 2012).

Nutrient deficiency stress

Deficiency in specific nutrients (N, P, K, Ca, Mg, S, or Fe) disrupts the functioning of the photosynthetic apparatus, decreasing PSII photochemical efficiency and modifying the values of ChlF parameters (Smethurst et al. 2005). Nitrogen (N) accessibility is the key factor limiting the growth of plants, being a component of all proteins and nucleic acids and other organic compounds. N deficiency modifies thylakoid membranes and disrupts their

functioning (Fig. 2e), leading to acceleration of chloroplast aging and plastoglobule formation (Wu et al. 2006). Nitrogen is also an important element in RuBisCO photosynthetic complexes, the Calvin–Benson cycle enzymes, chlorophyll, and carotenoids (Correia et al. 2005). Nitrogen deficiency contributes to reductions in transpiration, stomatal conductance, the chlorophyll and carotenoids content, and the concentration of soluble sugars (Huang et al. 2004). Insufficient nitrogen uptake also reduces the electron acceptor pool in PSII and decreases RuBisCO and phosphoenolpyruvate carboxylase (PEPCase) activity (Correia et al. 2005).

JIP-test analyses have been applied several times in studies dealing with nitrogen deficiency and the effects of poor nitrogen supply on PSII has been well described (Redillas et al. 2011, Li et al. 2012). Specifically, N deficiency led to a significant decrease of the density of reaction centers (Dudeja and Chaudhary 2005). Conversely, the positive effects of higher nitrogen treatments on PI values have been documented in soybean (van Heerden et al. 2004), maize (Li et al. 2012), and wheat (Zivcak et al. 2014b).

Phosphorus (P) is also essential for plant growth and development. Major deficits cause modifications in grain and thylakoid structure and in light-harvesting complexes absorbing PAR, thereby reducing PSII activity (Foyer and Spencer 1986). P deficiency also has a negative impact on NADPH regeneration, reduces the quantum yield and carboxylic efficiency of photosynthesis and the electron transport efficiency (Wu et al. 2006). The JIP-test has been successfully used to estimate the activity/efficiency of PSII in plants exposed to phosphorus deficiency stress (Kruger et al. 1997; Tsimilli-Michael and Strasser 2008). Indeed, various studies have demonstrated a correlation between JIP-test parameters and gas exchange orplant growth parameters (Strasser et al. 2000).

Potassium (K) plays a key role in cellular osmoregulation: its ions are necessary to retain the pH gradient across the thylakoid membrane (Rampino et al. 2006). P deficiency increases stomatal conductance resistance, limiting carbon dioxide diffusion through the stomata. In photosynthesis, potassium's role in the activation of numerous enzymes and in ATP synthesis is probably much more significant than its role in controlling stomatal functioning. However, little is known about the impact of P deficiency on photosynthetic apparatus efficiency and PSII functioning. Nevertheless, a decrease in values of some photosynthetic parameters, such as electron transport efficiency and maximum quantum yield of PSII, has been observed under P deficiency (Schweiger et al. 1996).

There are numerous studies that have used prompt ChlF parameters to analyze the effects on photochemical functions of other mineral deficiencies, such as calcium (Liu et al. 2009; Lauriano et al. 2006), magnesium (Smethurst et al. 2005), and iron (Molassiotis et al. 2006). As many nutrients have specific effects on PSII photochemistry, the question here is whether it is possible to identify nutrient deficiencies using chlorophyll fluorescence kinetics. Although this issue remains open, Kalaji et al. (2014a, b) were able to recognize deficiencies of the main nutrients in tomato using principal component analysis of data derived from prompt ChlF analysis.

Heavy metal stress

High levels of heavy metals disrupt the photosynthesis process, but the impact of particular heavy metal ions may be species specific (Antosiewicz 2005; Mishra and Dubey 2005). Photosystem I (PSI) is considered more tolerant of heavy metal impact than PSII (Romanowska et al. 2006; Tuba et al. 2010).

Cadmium (Cd) is one of the most toxic heavy metals and can accumulate in living organisms. Sources of Cd in the environment include phosphate fertilizers and industrial waste products (Romanowska et al. 2006; Kalaji and Łoboda 2007). However, Cd does not appear to affect the amount of photosynthetic pigments: research on oilseed rape *Brassica napus* L. seedlings grown in the presence of Cd for 2 weeks revealed no significant changes to the content of chlorophyll a, chlorophyll b, and carotenoids (Janeczko et al. 2005). Nevertheless, Cd does have a negative impact on the photochemical efficiency of the photosynthetic process. PSII is more sensitive to its impact than the PSI, indicating that Cd disrupts the PSII functions with greater intensity (Mallick and Mohn 2003). Cd affects both the donor and acceptor sides of PSII. On the donor side, it inhibits the OEC, while on the acceptor site it inhibits electron transport between Q_A^- and Q_B^- (Sigfridsson et al. 2004). Disruption of the electron transport chain is due to degradation of the LHCII oligomer. The presence of Cd ions also increases the heat dissipation of excitation energy—defined as non-photochemical quenching (Janeczko et al. 2005). A detailed analysis of ChlF records from oilseed rape revealed that Cd caused a decrease in specific energy flow per sample cross section. Specifically, decreases in RC/CS, ET_O/CS, and in the activity of OEC were observed (Janeczko et al. 2005). F_V/F_M appears to be the least sensitive Cd impact parameter, indicating the maximum quantum yield of PSII. Plant resistance to Cd is associated with the ability of "sweeping" ROS, launching protective mechanisms such as activation of antioxidant enzymes, in particular peroxidase (Ekmekçi et al. 2008), and the synthesis of antioxidant-active compounds, e.g., glutathione (Streb et al. 2008).

Lead (Pb) also has harmful effects on plants. The main sources of Pb in soil and plants are gas emissions from

coal-fueled power plants, fuel gases, and industrial technology (Mishra and Dubey 2005). Pb causes modification of respiration and increases ATP and the ATP/ADP ratio, a result of mitochondrial production of this high-energy compound (Romanowska et al. 2002). The decrease in the efficiency of photosynthesis in plants under Pb stress is a result of disruption of chloroplast ultrastructure and thylakoid membrane lipid composition, and a reduction of synthesis of chlorophyll and carotenoids (Sharma and Dubey 2005). Pb interrupts the uptake of nutrients (such as magnesium and iron) which, in turn, are essential for photosynthesis. Moreover, it causes the dissociation of the polypeptides OEC and the removal of Ca, Cl, and Mn compounds from this complex (Sharma and Dubey 2005; Romanowska et al. 2006). The intensities of fluorescence at I and P steps in the O–J–I–P induction curve of plants exposed to Pb stress decreased in relation to the control (Fig. 2f), and a peak (K) occurred (Kalaji and Łoboda 2007). The appearance of this point on the ChlF induction curve may be associated with electron transport inhibition between the OEC and the PSII reaction center (Strasser et al. 2004; Wu et al. 2008). Models of Pb stress suggest that energy absorption and dissipation within the PSII are high, while electron trapping and transport are reduced (Lazár and Jablonský 2009).

Limitations of prompt ChlF methods

Mathematical models for analysis of prompt ChlF kinetics, such as JIP-test, were developed exclusively as a biophysical tool for assessment of the cascade of chloroplast redox reactions at microsecond or millisecond scales. Nevertheless, even early studies generated interesting empirical knowledge on the relationship between the physiological status of the sample and the shape of fluorescence transient (Strasser et al. 2000). There have subsequently been many articles documenting the direct relationship between the physiological status of leaves and prompt ChlF transient. This reflects the fact (often neglected) that the measured signal is the mixture of many signals (see paragraph on PSII heterogeneity above) related to processes associated with adjusting of structure and function of photosynthetic apparatus to current metabolic needs or environmental conditions. Thus, several factors need to be carefully monitored to avoid erroneous and over-simplified interpretations (Evans 2009).

The simplicity and rapidity of the method together with misunderstanding of the basic principles has also led to incorrect applications. The use of integrative parameters such as performance index (PI) can be more useful than complex of specific biophysical parameters, which require a deeper understanding of photochemical processes to interpret the data correctly. Pros and Cons of the analysis of the OJIP transient by the so-called JIP-test are well discussed by Stirbet and Govindjee (2011). To avoid the mistakes in ChlF applications, all users are strongly encouraged to be familiar with practical aspects of measurements (reviewed in Kalaji et al. 2014a).

Concluding remarks

This review paper brings up-to-date information on the vast opportunities of the application of chlorophyll fluorescence technique in plant science, agricultural and ecological research. Measured signals of chlorophyll fluorescence and its statistical analysis (e.g., by JIP-test) can be used to predict, monitor, and identify stress in plants. Consequently, it could be applied in almost any ecological study of plants as a bioindicator. The versatility of ChlF measurements means they can be applied at the level of a single plant to grassland, cropland, and even marine ecosystems. However, this potential versatility emphasizes the need for more practical and conceptual studies that would allow scientists to draw reliable information about plant growth and health. Such an approach would not only lead to improvements in our understanding of the physiological basis of photosynthesis but could also contribute to efforts to understand and remediate the impacts of climate change on crop yields and food security.

Author contribution statement Kalaji M. H. and Jajoo A. created the idea of the manuscript and put the first items to be considered in this paper. Moreover, they edited the main lines of the text. Samborska I. A., Cetner M. D., and Łukasik I. developed the initial work suggested by the first two co-authors and added proper details and examples. Brestic M., Zivcak M., Oukarroum A., and Goltsev V. enhanced the text by adding the proper references, figures, and built up the discussion in this manuscript. Richard J. Ladle provided professional (biological) language corrections. We confirm that, all above-listed co-authors contributed equally to this work.

Acknowledgments The authors thank Prof. Govindjee for supporting the idea of this paper, reading, and commenting on the draft of this paper.

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