Genotypically Identifying Wheat Mesophyll Conductance Regulation under Progressive Drought Stress

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Photosynthesis limitation by CO2 flow constraints from sub-stomatal cavities to carboxylation sites in chloroplasts under drought stress conditions is, at least in some plant species or crops not fully understood, yet. Leaf mesophyll conductance for CO2 (g_m) may considerably affect both photosynthesis and water use efficiency (WUE) in plants under drought conditions. The aim of our study was to detect the responses of g_m in leaves of four winter wheat (Triticum aestivum L.) genotypes from different origins under long-term progressive drought. Based on the measurement of gas-exchange parameters the variability of genotypic responses was analyzed at stomatal (stomata closure) and non-stomatal (diffusional and biochemical) limits of net CO2 assimilation rate (A_N). In general, progressive drought caused an increasing leaf diffusion resistance against CO2 flow leading to the decrease of A_N, g_m and stomatal conductance (g_s), respectively. Reduction of g_m also led to inhibition of carboxylation efficiency (Vc_max). On the basis of achieved results a strong positive relationship between g_m and g_s was found out indicating a co-regulation and mutual independence of the relationship under the drought conditions. In severely stressed plants, the stomatal limitation of the CO2 assimilation rate was progressively increased, but to a less extent in comparison to g_m, while a non-stomatal limitation became more dominant due to the prolonged drought. Mesophyll conductance (g_m) seems to be a suitable mechanism and parameter for selection of improved diffusional properties and photosynthetic carbon assimilation in C3 plants, thus explaining their better photosynthetic performance at a whole plant level during periods of drought.

Keywords: photosynthesis, drought, mesophyll conductance, A_N/Ci, carboxylation efficiency, wheat

INTRODUCTION

At the global level, drought accompanied by low water availability in soils is considered the main environmental factor that limits plant growth and yield (Chaves et al., 2003; Nemani et al., 2003; Zhao et al., 2011). This combination may negatively affect the productivity of agricultural crops as well as natural ecosystems and the diversity of plant species (Zivcak et al., 2013). There are some
strategies aimed at maintaining water resources in soils and plants, e.g., improvement of crop water use efficiency (WUE; Wang et al., 2002; Condon et al., 2004) and photosynthesis itself, which may increase crop yields in the near future (Parry et al., 2002; Flexas et al., 2013).

A water deficit develops in plants when water losses by evapotranspiration are inadequately replaced by the water flow from soil. In a natural environment, a water deficit occurs progressively from a week to months, depending upon the characteristics of the soil where the plants are grown (Cano et al., 2014). Water deficiency triggers many responses at different levels (molecular to whole plant) of plants in conditions of water scarcity (Shao et al., 2009; Zivcak et al., 2014) that involve different survival strategies (such as stress escape, avoidance or tolerance), adaptive changes and deleterious effects which can all develop even in parallel (Barnabás et al., 2008). They also include the production of many biological macro- and micro-molecules, such as nucleic acids (DNA, RNA, microRNA), proteins, carbohydrates, lipids, hormones, ions, or mineral elements (Shao et al., 2006). These responses to external limiting factors can vary and are genotype- and species-related (Rampino et al., 2006), including the length, intensity and duration of water stress (Araus et al., 2002), plant age and ontogeny (Zhu et al., 2005), light and temperature (Gallé et al., 2009), intensity of previous stresses (Flexas et al., 2009), as well the application of successive drought and recovery cycles (Gallé et al., 2011). Moreover, under natural conditions, plants are often exposed to multiple stress factors that influence photosynthesis and growth (Lu et al., 2003). The combination of drought with other abiotic stress factors, such as intense light, salinity or heat, considerably increases the photoinhibition of photosynthesis (Shao et al., 2006; Yan et al., 2013).

The impact of drought on photosynthesis can basically be divided into two groups: (i) a direct effect, which increases the restriction of the CO2 diffusion pathway via stomata, as intercellular airspaces leading to the mesophyll cells that cause a decline in CO2 availability for Rubisco (Cornic et al., 1989; Chaves, 1991; Flexas et al., 2004a,b, 2007; McDowell, 2011), (ii) an indirect effect, such as alterations in the biochemistry and metabolism of the photosynthetic apparatus, membrane permeability (aquaporins) (Lawlor and Cornic, 2002; Chaves et al., 2009) and the promotion of oxidative stress (Aranda et al., 2012).

Indeed, restricted CO2 diffusion from the atmosphere to the site of carboxylation is the main reason for decreased photosynthesis under water stress conditions caused by both the stomatal and mesophyll limitations (Centritto et al., 2003; Flexas et al., 2004a,b; Grassi and Magnani, 2005; Zivcak et al., 2014). Stomata are the primary component of the CO2 diffusional pathway, which limits water loss. Under prolonged drought, they also limit the CO2 supply inside the leaves (Martorell et al., 2014). In C3 plants, low g_m reduces water loss from drying plants to save water via a rapid and effective survival strategy. The stomata response could vary in degree, becoming more pronounced with the increasing severity of a stress (Zivcak et al., 2013). The net CO2 assimilation rate (A_N) is usually reduced by water deficit due to not only stomatal closure but also non-stomatal processes (Medrano et al., 2002) such as decreased g_m (Flexas et al., 2008).

According to Fick’s first law of diffusion, A_N is determined as follows: A_N = g_m(C_a - C_i) = g_m(C_i - C_c), where C_a, C_i, and C_c are the CO2 concentrations in the atmosphere, sub-stomatal cavities and carboxylation site of Rubisco, respectively (Long and Bernacchi, 2003). Previous works usually stated that g_m is large and constant (therefore, C_i = C_c). However, at present, there are many lines of evidence suggesting that the CO2 concentration in chloroplasts is significantly lower than in sub-stomatal cavities because of the finite value of g_m (von Caemmerer and Evans, 1991; Niinemets et al., 2009). Although g_m is rather small, it markedly regulates C_i and hence limits leaf photosynthesis (Di Marco et al., 1990; Harley et al., 1992; Loreto et al., 1992; Warren and Adams, 2006).

The mesophyll conductance indicates the conductance for CO2 flowing from the intercellular air spaces to the site of carboxylation in the chloroplasts of mesophyll cells and includes the quite complicated pathways of the cell wall, plasma membrane, chloroplast envelope, and stromal thylakoids. It involves gas phase resistance among intercellular air spaces and liquid phase resistance from the cell wall to stroma (Evans et al., 2009). Recent studies show a crucial role for g_m in the regulation of photosynthesis, and it has already been assumed that g_m represents up to 40% of the CO2 diffusional limitations to whole photosynthesis (Warren, 2008).

Currently, there are many studies showing decreased g_m during a progressive leaf water deficit. Recent studies (Rousset et al., 1996; Flexas et al., 2004a, 2006; Delfine et al., 2005; Galmés et al., 2007, 2011; Tomás et al., 2013; Niinemets and Keenan, 2014) clearly confirm that drought in plants may significantly limit g_m. Nevertheless, it remains unknown which mechanisms are responsible for the reduction of g_m. Any changes in g_m during low soil water availability may potentially play an important role in the regulation and control of photosynthesis (Flexas et al., 2014). It is hypothesized that a crop under drought stress should reach low stomatal conductance (g_s), which can reduce water loss but consequently maintains a high intensity of carbon fixation. This is only possible when the CO2 concentration in chloroplasts (C_c) remains high as a result of improved g_m (Flexas et al., 2012).

The high sensitivity of g_m to different environmental factors has already been shown with the reactions occurring in a wide time range, from minutes to hours (Pons and Welchen, 2003; Flexas et al., 2012). Recent reviews have already highlighted the effects of environmental conditions, such as increased and decreased CO2 concentration around leaves (Harley et al., 1992; Centritto et al., 2003), exogenous application of ABA and polyethylene glycol (Flexas et al., 2006), high altitude (Vitousek et al., 1990), low light (Laisk et al., 2005), low...
nitrogen availability (Warren and Adams, 2006), low and high temperatures (Bernacchi et al., 2002; Pons and Welchen, 2003; Yamori et al., 2006), or viral infections (Sampol et al., 2003). There is also increasing evidence to suggest a significant role for aquaporins in the control of membrane permeability to CO₂, which are also limiting factors of gₑ in C₃ plants (Heckwolf et al., 2011; Sade et al., 2014). In particular, gₑ is also determined by the variability of leaf structural traits, such as leaf thickness, cell packing, shape, and wall thickness (Tosens et al., 2012; Tomás et al., 2013; Muir et al., 2014).

The decrease in AN as a consequence of water stress is also commonly analyzed in terms of the stomatal and non-stomatal limitations (Grassi and Magnani, 2005). However, the dynamics between the stomatal and non-stomatal limitations during drought remain unclear (Lawlor and Cornic, 2002; Loreto and Centritto, 2008). In previous decades, valuable studies of sufficient quantity accumulated on the effect of drought on gₑ. Indeed, inter-specific genotypic differences in gₑ have already been found for several species, e.g., Vitis vinifera (Tomás et al., 2013), Hordeum vulgare (Barbour et al., 2010), Castanea sativa, Solanum lycopersicum (Galmés et al., 2011), Oryza sativa (Gu et al., 2012), and Triticum aestivum (Jahan et al., 2014).

The aim of this work was to perform an eco-physiological analysis of the main diffusional limits to leaf photosynthesis in wheat under a long-term progressive drought by determination of the dynamics and proportion of mesophyll vs. stomatal limitation changes and their sensitivity to water scarcity in four winter wheat genotypes of different geographical proveniences.

MATERIALS AND METHODS

Biological Material and Cultivation

The outdoor pot experiment was conducted in the experimental cage of the Department of Plant Physiology, Slovak University of Agriculture in Nitra. Seeds of four winter wheat (T. aestivum L.) genotypes (Šamorinska from Slovakia, GK Forrás from Hungary, Pehlivan from Turkey and Piopio-4 from Mexico) were selected on the basis of their (i) geographical origin (European genotypes–Middle to South Europe vs. Latin America), (ii) historical view of wheat breeding (Šamorinska as a landrace vs. GK Forrás, Pehlivan, and Piopio-4 as modern genotypes) and (iii) different mechanism of WUE regulation under drought conditions. They were obtained from the Gene bank in Plant Production Research Institute in Piestany (Slovakia). The seeds were sown in plastic pots (15 l volume) filled with a mixture of horticultural substrate and clay soil in 1:1 ratio. The substrate of pH 7.3 contained 40.08 mg kg⁻¹ Nₐₙ, 206.5 mg kg⁻¹ P, 590 mg kg⁻¹ K, and 3.73% of humus. Plants were grown in a natural environmental conditions and were regularly irrigated to maintain the optimum field water capacity during whole experiment. The foliar application of liquid fertilizers with macro- and micro-nutrients was carried out in the early spring time. At the growth stage of inflorescence emergence (BBCH-51, Zadoks et al., 1974), the progressive dehydration of soil and plants in pots was induced by a withholding watering for 21 days. The responses of photosynthesis and water status to the induced water stress were measured simultaneously from gas exchange and leaf RWC data. The leaf hydration range was used for differentiation of the water stress level, and the data were clustered into three groups, e.g., well-watered plants (WW; RWC = 80–100%), mild water stress (MS; RWC = 60–80%) and severe (SS; RWC = 40–60%) water stress. After the dehydration period watering of plants continued optimally. Climatic data (average daily temperature and daily total precipitation; Figure 1) were obtained from the meteorological station of Horticulture and Landscape Engineering Faculty in SUA Nitra, localized in neighborhood of the experimental site.

Gas Exchange and Chlorophyll a Fluorescence Measurements

Gas exchange measurements were made daily on fully expanded flag leaves of control and stressed plants from the beginning of the dehydration process to its terminal phase when the stomata were fully closed.

The AN/C₄ response curves of plants from each genotype were measured on a daily basis using the open gas-exchange system Li-6400XT (Li-Cor Inc., Lincoln, Nebraska, USA) with an integrated fluorescence chamber head Li-6400-40 (Li-Cor Inc.). Gas-exchange and chlorophyll a fluorescence parameters were measured in light-adapted leaves at saturation PPFD set up at 1500 µmol m⁻² s⁻¹ with 10% blue light to maximize stomatal aperture. Leaf temperature was kept at 21°C and relative air humidity was maintained between 60 and 70% during all measurements. Gas exchange and chlorophyll a fluorescence were first measured after reaching steady-state at 380 µmol CO₂ mol⁻¹ air surrounding the leaf (Cₐ). Subsequently, Cₐ was decreased stepwise until 50 µmol mol⁻¹ and then increased stepwise until 1500 µmol CO₂ mol⁻¹. The number of different Cₐ values used for the AN/C₄ response curves was 12, and the time between the two consecutive measurements at different Cₐ values was maximal 4 min.

The actual photochemical efficiency of photosystem II (Φₚₛₛᵢ) was assessed following the procedures of Genty et al. (1989) based on the measurements of actual (Fₐ) and maximal (Fₚₐₚ) fluorescence during pulse light saturation (intensity 8000 µmol m⁻² s⁻¹) and calculated as follows:

\[ \Phi_{PSII} = (F_{m'} - F_i)/F_{m'} \]

The electron transport rate (Jₑ) was calculated as:

\[ J_e = \Phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \]

where PPFD is the photosynthetically active photon flux density, \( \alpha \) is leaf absorbance (0.85), and \( \beta \) is the partitioning of absorbed quanta between the PSII and PSI. The method of Valentiniet al. (1995) was used to determine the product of \( \alpha \beta \) from the relationship between \( \Phi_{PSII} \) and \( \Phi_{CO2} (\Phi_{CO2} = (AN + R_d)/PPFD) \), where \( R_d \) is the daytime respiration rate determined by the Laisk method (Laisk, 1977) (see next section) obtained by varying \( C_a \) (11 different values) under non-photorespiratory conditions in an atmosphere containing less than 1% O₂, a leaf temperature of 21°C, saturation PPFD (1500 µmol m⁻² s⁻¹) and a relative humidity of 75%.
Flow of CO₂ out and into the leaf cuvette was determined for the range of C<sub>a</sub> values used with photosynthetically inactive leaves (obtained by heating) of each genotype enclosed in the chamber; the correction was used for the calculation of CO₂ fluxes (Flexas et al., 2007).

Leaf-intrinsic WUE was calculated as A<sub>N</sub> to g<sub>s</sub> ratio from gas-exchange measurements of C<sub>a</sub> at 380 µmol CO₂ mol⁻¹ air and saturating light.

**Calculation of g<sub>m</sub>**

The mesophyll conductance for CO₂ (g<sub>m</sub>) was estimated from simultaneously measured gas-exchange and chlorophyll a fluorescence parameters of varying C<sub>a</sub> according to Harley et al. (1992):

\[
g_{m} = \frac{A_{N}}{C_{i}} - \frac{\Gamma_{*}[I_{f} + 8(A_{N} + R_{d})]}{4(A_{N} + R_{d})}
\]

where A<sub>N</sub>, I<sub>f</sub> and C<sub>i</sub> were obtained during the dehydration from gas-exchange measurements of C<sub>a</sub> at 380 µmol CO₂ mol⁻¹ air and saturating light. The chloroplastic CO₂ compensation point (Γ<sup>*</sup>) and daytime respiration rate (R<sub>d</sub>) were estimated using the method of Laisk (1977). Several A<sub>N</sub>/C<sub>i</sub> response curves were measured at three different PPFDs (50, 150, and 300 µmol m⁻² s⁻¹) and six different C<sub>i</sub> levels (250 to 50 µmol mol⁻¹) for each genotype in well-watered plants. The intersection point of the linear regression of A<sub>N</sub>/C<sub>i</sub> response curves was used to determine the apparent CO₂ compensation point, C<sup>*</sup><sub>i</sub> (x-axis) and R<sub>d</sub> (y-axis). C<sup>*</sup><sub>i</sub> was used as a proxy for Γ<sup>*</sup> (Warren and Adams, 2006). The measured data of R<sub>d</sub> and Γ<sup>*</sup> which were used for the calculation of g<sub>m</sub> are shown in Table 1.

**Calculation of V<sub>c</sub>max**

The maximal in vivo carboxylation activity of Rubisco (V<sub>c</sub>max) was calculated from the gas exchange measurement by the data fitting procedure of the initial slope of the A<sub>N</sub>/C<sub>i</sub> curve

| Genotype         | Γ<sup>*</sup> (µmol mol⁻¹) | R<sub>d</sub> (µmol m⁻² s⁻¹) |
|------------------|--------------------------|-----------------------------|
| GK Forrás        | 36.38 ± 2.58             | 2.18 ± 0.07                 |
| Peřívan          | 34.86 ± 2.61             | 2.13 ± 0.05                 |
| Piopio-4         | 35.15 ± 1.01             | 2.14 ± 0.06                 |
| Šamorínska       | 34.08 ± 1.70             | 2.08 ± 0.04                 |

Data represent the means of set measurements performed by the Laisk method ± S.E. (p = 3). (C<sub>i</sub> < 300 µmol mol⁻¹):

\[
A = \frac{V_{c}^{max} \cdot (C_{i} - \Gamma^{*})}{C_{i} + K_{c} \cdot (1 + \frac{O}{K_{o}})}
\]

where A is the net assimilation rate limited by Rubisco activity, and K<sub>c</sub> and K<sub:o</sub> are the Michaelis-Menten constants of Rubisco activity for RuBP carboxylation and oxygenation, respectively. K<sub>c</sub> and K<sub:o</sub> are assumed to be 404.9 µmol mol⁻¹ and 278.4 mmol mol⁻¹ at 25°C, respectively, according to Bernacchi et al. (2001). Oxygen concentration in chloroplasts (O) was assumed to be 210 mmol mol⁻¹.

**Estimation of Relative Limitation to Photosynthesis**

The limitation of photosynthesis based on g<sub>s</sub> and g<sub>m</sub> was estimated as potential rate of photosynthesis assuming these conductance values were infinite or measured, respectively (Farquhar and Sharkey, 1982). A<sub>N</sub>/C<sub>i</sub> curves were used to separate and estimate the stomatal and non-stomatal limitations to photosynthesis. To assess an effect of dehydration on CO₂ assimilation, the photosynthetic limitations were partitioned into the components related to stomatal and mesophyll conductance.
according to Warren et al. (2003) and calculated as follows:

\[
L_S = 100 \cdot \frac{A_{Ci} - A_{Ca}}{A_{Ci}},
\]

\[
L_M = 100 \cdot \frac{A_{Cc} - A_{Ca}}{A_{Cc}},
\]

where \(L_S\) and \(L_M\) are the relative stomatal and mesophyll limitation of \(A_N\), respectively, \(A_{Ca}\) is the light-saturated rate of photosynthesis at \(C_a = 380 \mu\text{mol m}^{-2}\text{s}^{-1}\) (\(g_s\) and \(g_m\) as measured), \(A_{Ci}\) is the light-saturated rate of photosynthesis at \(C_i = 380 \mu\text{mol m}^{-2}\text{s}^{-1}\) (assuming \(g_s\) was infinite and \(g_m\) was measured), and \(A_{Cc}\) is the light-saturated rate of photosynthesis at \(C_c = C_i\) (assuming \(g_m\) was infinite and \(g_s\) was measured).

**Relative Water Content**

The leaf relative water content (RWC) was determined as:

\[
RWC = \frac{(FW - DW)}{(TW - DW)} \cdot 100
\]

The leaf disc was cut out from the central part of a measured leaf. Fresh weight (FW) was determined immediately after the gas exchange measurement. Turgid weight (TW) was obtained after 12 h of hydration, when a leaf disc was kept in distilled water at 4°C in the dark. Dry weight (DW) was measured after drying the leaf disc at 80°C for 24 h.

**Statistical Analyses**

The experiment with wheat plants in pots was established by block method with a completely randomized design of experimental plots. All analyses were performed using the Statistica v. 10 software (StatSoft Inc., Tulsa, Oklahoma, USA) and the graphics software SigmaPlot version 11.0 (Systat Software Inc., San Jose, California, USA). Analysis of variance was performed between the different levels of drought (well-watered, MS, SS) and the genotypes was tested by the HSD test. All analyses were performed using the Statistica v. 10 software (StatSoft Inc., Tulsa, Oklahoma, USA) and the graphics software SigmaPlot version 11.0 (Systat Software Inc., San Jose, California, USA). Analysis of variance was performed between the different levels of drought (well-watered, MS, SS) and the genotypes was tested by the HSD test.

**RESULTS**

Climatic conditions at the experimental site are shown in Figure 1. Average daily temperature during the growing season (October 5, 2010 to July 4, 2011) was 7.4°C with the sum of precipitation of 373.9 mm. The sum of active daily temperatures (above 10°C) per growing season was 1658°C. The average daily temperature during the drought treatment was 20.03°C.

Significant differences among the investigated wheat genotypes grown in WW conditions were found for \(A_N\), \(g_s\), \(g_m\), and \(V_{cmax}\). The \(A_N\) and \(g_s\) varied from 26.39 to 28.64 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) and 0.50 to 0.43 \(\mu\text{mol m}^{-2}\text{s}^{-1}\), respectively. Differences among genotypes for WUEi were non-significant \((p > 0.05)\) and varied from 56.87 to 64.52 \(\mu\text{mol CO}_2 \text{ mol}^{-1}\) \(\text{H}_2\text{O}\). Genotype Pehlivan reached the highest value for these parameters (Table 2). Genotypic variation in \(g_s\) (c.v. 12%) explained 7% of the observed variability in \(A_N\) under WW conditions (Table 3). Mesophyll conductance \((g_m)\) in WW plants varied nearly 3-fold among all genotypes, from 0.24 to 0.73 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) \((p < 0.001)\). The highest value for \(g_m\) was observed in the genotype Pehlivan.

Significant reductions in \(A_N\), \(g_s\), and \(g_m\) were observed under progressive dehydration from WW conditions (Figure 2, Tables 2, 3). Under the MS conditions, significant genotypic differences were found in \(A_N\) and \(g_s\), which varied from 16.01 to 19.35 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) and 0.29 to 0.42 \(\mu\text{mol m}^{-2}\text{s}^{-1}\), respectively. Thus, the average 1.5-fold reduction of \(A_N\) was accompanied by an almost 20% reduction of \(g_s\) and a 3.5-fold reduction of \(g_m\). The highest stomatal sensitivity to the decline in RWC was observed in genotype Šamorínska, while the highest sensitivity of \(g_m\) to RWC was found in genotype Pehlivan. Under severe water stress (SS) conditions, \(g_s\) declined below 0.15 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) in all genotypes, with the most pronounced reduction in GK Forrás. However, we should be noted that in genotypes Pipioio-4 and Šamorínska originating from Mexico and Slovakia (Šamorínska is a Slovakian landrace), respectively, the dehydration cycle was faster (11 days), causing the \(g_s\) to drop below 0.08 \(\mu\text{mol m}^{-2}\text{s}^{-1}\), while in genotypes Pehlivan and GK Forrás from Turkey and Hungary, similar \(g_s\) values (0.09 and 0.15 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) were reached after 15 and 16 days of dehydration, respectively.

The reduction of leaf RWC resulted in the decline of \(g_m\) (0.05–0.06 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) with non-significant \((p > 0.05)\) genotypic differences. The \(g_s\) and \(g_m\) reductions resulted in the reduction of \(A_N\) (Table 2). Then, the reduction of \(g_s\) relative to \(A_N\) in genotype GK Forrás under drought condition significantly \((p < 0.001)\) increased WUEi. Finally, under SS conditions, the genotypic variation in \(g_s\) (c.v. 49%) explained 12% of the observed variability in \(A_N\) (Table 3).

There was a clear polynomial decline in \(g_s\) induced by stomatal closure in the plant response to progressive drought, showing the same trends for genotypes GK Forrás, Pehlivan, and Pipioio-4 (Figures 2A–C), with the exception of genotype Šamorínska (Figure 2D), which showed almost linear decline of \(g_s\). This result indicates a high stomatal sensitivity of landrace genotype to water stress, confirming that stomata were completely closed after 11 days of dehydration.

As shown in Figure 3, the \(A_N\) was positively correlated with \(g_m\) under progressive dehydration in all genotypes \((r^2 = 0.890)\) for Pehlivan to 0.924 for Šamorínska; \(p < 0.001)\). A significant decline in \(A_N\) in response to reduced \(g_m\) was observed under the transition from WW to MS conditions. Under SS conditions, a strong reduction of \(g_m\) (below 0.15 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) resulted in a progressive decline of \(A_N\); however, this was still above the \(\text{CO}_2\) compensation point in all genotypes. The largest slope of the \(A_N/g_m\) relationship was observed in the Pipioio-4 genotype, where we conclude that the drought stress had a greater impact on \(g_m\) compared to \(A_N\).

Analysis of the in vivo maximal carboxylation activity of Rubisco \((V_{cmax})\) revealed the genotypic variability \((p < 0.05)\) only under well-watered conditions (Figure 4), with the changes ranging from 88.14 ± 6.3 to 108.44 ± 8.2 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) for genotypes Šamorínska and Pehlivan, respectively. Water stress (MS and SS) significantly \((p < 0.01)\) reduced \(V_{cmax}\), but without any genotypic difference. The mean level of \(V_{cmax}\) was 74.8 ± 5.4 and 39.12 ± 1.2 \(\mu\text{mol m}^{-2}\text{s}^{-1}\) both in MS and SS,
TABLE 2 | The net CO2 assimilation rate (AN; μmol m−2 s−1), stomatal conductance to H2O (gs; mol H2O m−2 s−1), mesophyll conductance to CO2 (gm; mol CO2 m−2 s−1) and leaf-intrinsic water use efficiency (WUE) calculated as AN/gs ratio; μmol CO2 mol−1 H2O) in flag leaves of four wheat genotypes under well-watered (WW; RWC = 80–100%), mild stressed (MS; RWC = 60–80%) and severe stressed (SS; RWC = 40–60%) conditions.

| Genotype | AN (μmol m−2 s−1) | gs (μmol mol−1 s−1) | gm (μmol m−2 s−1) | WUE |
|----------|------------------|------------------|------------------|-----|
| GK Forrás WW | 27.61 ± 1.67Ab | 0.43 ± 0.06Ba | 0.45 ± 0.04Ba | 64.52 ± 8.45Ab |
| MS | 16.01 ± 2.25Ab | 0.39 ± 0.08Ab | 0.16 ± 0.06Ab | 42.18 ± 5.51Bc |
| SS | 7.12 ± 2.11Abc | 0.09 ± 0.06Bc | 0.06 ± 0.02Bc | 124.22 ± 30.79Ab |
| Pehlivan WW | 28.64 ± 1.82Ab | 0.50 ± 0.04Ab | 0.73 ± 0.09Ab | 56.87 ± 6.32Ab |
| MS | 19.35 ± 3.38Ab | 0.42 ± 0.04Ab | 0.16 ± 0.08Ab | 48.63 ± 9.83Ab |
| SS | 4.98 ± 2.19Cc | 0.15 ± 0.04Ac | 0.06 ± 0.03Ac | 52.61 ± 18.67Ab |
| Piopio-4 WW | 25.85 ± 1.74Bb | 0.46 ± 0.06Bb | 0.24 ± 0.02Bb | 57.00 ± 3.79Ab |
| MS | 16.16 ± 2.25Bb | 0.37 ± 0.04Bb | 0.09 ± 0.02Bb | 46.26 ± 9.01Bb |
| SS | 5.65 ± 2.32Cc | 0.11 ± 0.08Bc | 0.05 ± 0.01Ac | 33.88 ± 6.45Bb |
| Šamorínska WW | 26.39 ± 1.10Bb | 0.45 ± 0.06Bb | 0.44 ± 0.07Bb | 58.79 ± 6.72Ab |
| MS | 17.00 ± 2.43Abb | 0.29 ± 0.06Cb | 0.12 ± 0.03Cb | 64.11 ± 18.00Ab |
| SS | 8.44 ± 2.11Ac | 0.13 ± 0.04Cbc | 0.06 ± 0.01Ac | 61.42 ± 11.97Bb |

The data are the means ± S.E. (n = 12–20). S.E.=standard error. Superscript: large letters (A,B,C) denote significant differences at p < 0.05 obtained by Duncan’s post hoc test among all wheat genotypes at a given stress level (WW, MS or SS), and small letters (a,b,c) indicate statistical differences among all stress levels for a given genotype.

which constituted ~0.5-fold and 2.5-fold decline for MS and SS, respectively.

Analysis of the fast AN–C4 response curve showed that the gm calculated via the method of Harley et al. (1992) was not constant along the range of C4 values employed in this study (Figure 5). We observed the obviously known three-phase course of gm changes to varied C4 values. A strong sensitivity of gm at low C4 concentrations was observed in the first part of the response curve (C4i from ~80 to 200 μmol mol−1 air). After reaching an inflection peak of gm at C4 concentrations from 200 to 400 μmol mol−1 air, the gm values declined exponentially under the value of 0.1 μmol m−2 s−1 at high C4. The maximal sensitivity of gm to increased C4 was observed in Pehlivan (Figure 5B) with a 16-fold reduction of gm observed until the steady-state level was reached. The weak sensitivity of gm to increased C4 (only ~4-fold decline) was observed in the Piopio-4 genotype (Figure 5C). The highest genotypic differences in the sensitivity of gm to C4 variations were observed at low C4i concentrations (GK Forrás and Pehlivan with relatively lower gm and Piopio-4 and Šamorínska with relatively higher gm). Water stress reduced the sensitivity of gm to C4 changes in all of the investigated genotypes. During the transition from the mild to severe water stress, the mechanism responsible for the gm reaction was clearly inhibited, and gm did not react as fast as in the case of well-watered plants. The gm was negatively affected under SS conditions in all genotypes when the response to altered C4 was inhibited. Our results support the suggestions of others that mild to severe drought strongly influences the mechanism of gm regulation (Figure 5).

As shown in Figure 6, a close relationship between gm and gs was observed in all genotypes and stress levels (r2 = 0.77; p < 0.001). During the transition state from WW to MS conditions, the 1.5-fold reduction of gs was accompanied by a 3-fold decline of gm. A further increase in water stress up to SS conditions resulted in progressive stomatal closure and a reduction of gs accompanied by only small changes in gm. However, the transition from WW to MS affected both gs and gm in approximately the same measure. Thus, the final gm/gs relationship was linear. The highest slope of gm/gs was identified for genotype Pehlivan, while the lowest was identified for Piopio-4.

Based on the analyses of the A4/C4 response curves measured on a daily basis during the experiment, the stomatal and mesophyll limitation ratio was calculated (Figure 7). After the determination of both limitations in all genotypes, genotypic differences in the limitations were evaluated. The observed
FIGURE 2 | Responses of stomatal conductance to $\text{H}_2\text{O}$ ($g_s$; mol H$_2$O m$^{-2}$ s$^{-1}$) and mesophyll conductance to CO$_2$ ($g_m$; mol CO$_2$ m$^{-2}$ s$^{-1}$) to the relative water content (RWC; %) of flag leaves in four wheat genotypes (A, E) GK Forrás, (B, F) Pehlivan, (C, G) Piopio-4 and (D, H) Šamorínska. The points represent individual measurements of leaves. Symbols: square-GK Forrás, circle-Pehlivan, up-triangle-Pio, down-triangle–Šamorínska; empty symbols–well-watered (RWC 80–100%) plants, gray symbols- mild stress (RWC 60–80%) and black symbols-severe stress (RWC 40–60%). The coefficients of determination ($r^2$) and significance level ($p$) as well as the lines of polynomial quadratic (A–D) and polynomial cubic (E–H) regressions are shown.

differences could tell us more about drought response reactions and could also help determine which limitation is more crucial for the regulation of photosynthesis during drought.

From the first day of the experiment, we assessed the initial values of stomatal ($L_S$) and mesophyll ($L_M$) limitations as a percentage (Figure 7). As drought progressed and leaf water deficit increased, both $L_S$ and $L_M$ increased simultaneously, but the dynamics of the increase became uneven. $L_S$ began to increase to a less extent than $L_M$. The maximal value of $L_S$ (22.53%) was reached in stressed plants of the old Slovak genotype Šamorínska. However, this is not a crucial value that limits leaf photosynthesis. Therefore, we suggest that $L_S$ did not play as important a role in comparison with $L_M$ in dehydrated plants of all selected genotypes. $L_M$ predominated in three genotypes (Šamorínska, GK Forrás and Piopio-4). Although the $L_S$ of Pehlivan was higher than $L_M$ in the first period of dehydration, it changed after $L_M$ dominated over $L_S$. In genotype Piopio-4, $L_M$ was mostly disabled by drought in comparison with other genotypes. It obtained very high initial values (31.91%) and increased even further with a culmination at 69.2% as the drought progressed. Additionally, a great impact of water deficit caused a significant increase in $L_M$ and was found in dehydrated plants of Pehlivan (76.2%) and GK Forrás (77.6%).

**DISCUSSION**

Soil water scarcity is the main limiting factor for crop growth and yield worldwide. Despite the increased knowledge over the past decade on the effects of water stress on photosynthesis, there is still a controversial debate whether water stress limits $A_N$ primarily by stomata closure (stomata limitation) or mesophyll limitation (diffusional and metabolic). A general response of plant tissues to soil water deficit is the decline of relative water content (RWC). This depends on the strength and duration of drought stress (Chaves et al., 2009). The withholding of water resulted in the reduction of stomatal conductance ($g_s$) as a consequence of stomatal closure (Table 2, Figure 2) with significant genotypic differences (Table 3). The higher stomata
FIGURE 3 | The net CO₂ assimilation rate ($A_N$, $\mu$mol CO₂ m⁻² s⁻¹) response to mesophyll conductance ($g_m$, mol CO₂ m⁻² s⁻¹) in (A) GK Forrás, (B) Pehlivan, (C) Piopio-4 and (D) Šamorínska genotypes under progressive drought conditions. The points represent individual measurements of leaves. The symbols are the same as in Figure 2. The coefficients of determination ($r^2$) and significance level ($p$) as well as the line of logarithmic regressions are shown in the plot.

FIGURE 4 | The maximum rate of carboxylation of Rubisco ($V_{c_{\text{max}}}$; $\mu$mol CO₂ m⁻² s⁻¹) calculated from the gas-exchange data measured in the leaves of four wheat genotypes at different levels of drought stress. Vertical bars are the means of 9–20 individual measurements per treatment ± S.E. Different letters indicate significant differences among genotypes ($p < 0.05$) based on Duncan’s post hoc test at one stress level; **Indicates significant differences among stress treatments ($p < 0.01$) based on the HSD test in one genotype.

sensitivity to RWC decline found in the genotype Šamorínska is the result of rapid water loss from leaf tissues (Figure 2D). As observed from our experimental data, modern genotypes reacted to drought by a slow reduction of $g_s$ at the initial phase of dehydration, probably due to better osmotic adjustment and/or a deeper and more efficient root system (Wasson et al., 2012).

The reduction in $A_N$ resulting from decreased RWC was significantly correlated with a decline in $g_m$. This response is similar to those observed in many studies, and it is thought to be the general acclimation response of plants to drought (Cornic et al., 1989; Chaves, 1991; Cornic, 2000; Flexas et al., 2006). Under the gradual dehydration induced by withholding watering in plants, a highly significant relationship ($r^2 = 0.93$; data not shown) between the RWC decline and the reduction in $A_N$ was observed. Flexas et al. (2006) summarized their own results and compared them with others to reach a compromise in order to determine what limits $A_N$ more, stomata closure or metabolic impairments in the mesophyll. They noted that the reduction of CO₂ supply from the atmosphere to chloroplasts was the main factor that decreased $A_N$ under drought conditions. However, metabolic impairments occurred as well, but only during stronger water stress when $g_s$ dropped below 0.10 mol H₂O m⁻² s⁻¹.

In our study with well-watered wheat plants, the observed $g_m$ corresponded to the $g_m$ level for wheat as found in many published works (Tazoe et al., 2009, 2011; Jahan et al., 2014; Sun et al., 2015). Interestingly, a wide interval and significant genotypic differences in $g_m$ (from 0.24 to 0.73 mol m⁻² s⁻¹) (Tables 2, 3) may be the result of both the differences in Rubisco activity and the anatomical properties of leaves, respectively (Evans et al., 1994, 2009; Medrano et al., 2002; Parry et al., 2002; Flexas et al., 2006; Niinemets et al., 2009; Tomás et al., 2013; Muir et al., 2014). The role of aquaporins in the transport of CO₂ and thus the regulation of $g_m$ are also essential (Hanba et al., 2004). Inter-specific variations in $g_m$ were also previously reported in a number of publications (Ether and Livingston, 2004; Niinemets et al., 2009; Tomás et al., 2013; Niinemets and Keenan, 2014).

Based on the data analyses, a strong relationship was observed in our measurements between $A_N$ and $g_m$ (Figure 3). The $g_m$ decreased simultaneously as $A_N$ declined, which was caused by enhanced water scarcity. This trend was found for each of the studied wheat genotypes. This observed strong correlation demonstrates a well-known fact about the substantial regulation

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of $g_{m}$ that is directly connected to $A_{N}$ and thus represents the main factor underlying diffusive limitation for CO$_2$ from the internal sub-stomatal cavities to the site of carboxylation (Tezara et al., 1999). Ultimately, due to this significant relationship, we could also consider $g_{m}$ as the main factor that limits photosynthesis (Lawlor and Cornic, 2002) and plays a crucial role in the entire metabolism within the leaf mesophyll (Flexas et al., 2012).

Previously, one group of researchers argued that the decline in $A_{N}$ occurs as a direct consequence of stomata closure, which restricts further CO$_2$ diffusion from the intercellular spaces to the sites of carboxylation (Sharkey, 1990; Chaves, 1991; Cornic, 2000). On the other hand, Tezara et al. (1999) suggested that the decline of $A_{N}$ is due to the impairment of ATP and RuBP synthesis and low ATP content, rather than stomata limitation. Another factor could be any of the processes of the Calvin cycle, although it is still not clear which of these might be involved. Moreover, drought is able to damage and influence processes involved in RuBP regeneration, e.g., activities of key enzymes of the Calvin cycle, such as fructose-1,6-bisphosphate phosphatase, NADP:glyceraldehyde-3-phosphate dehydrogenase, ribulose-5-phosphate kinase, or 3-phosphoglycerate kinase (Flexas et al., 2004a).

It has been established that $g_{m}$ is a finite variable (Niinemets et al., 2009). By simultaneously measuring gas exchange and chlorophyll $a$ fluorescence, we exposed a substantial inhibition of $g_{m}$ during the development of water stress. It has been shown that $g_{m}$ is extremely sensitive to drought; photosynthesis in water-stressed conditions is considerably reduced (Grassi and Magnani, 2005; Flexas et al., 2006, 2007). In accordance with this, our results confirmed the differences in the kinetics of mesophyll limitation during photosynthesis (Figure 3). The genotypes Pehlivan and Piopio-4 differed the most in this regard (Figures 3B,C).

It is also well-known that $g_{m}$ controls the metabolic and anatomical properties of leaves during photosynthesis. Both the amount and activity of Rubisco are crucial in the control of $g_{m}$ (Niinemets et al., 2009). Therefore, we would expect a large inhibition of the maximal in vivo carboxylation activity of Rubisco ($V_{C_{max}}$) due to prolonged dehydration, which has already been established. During mild and severe stress conditions, drought induced a significant (2.5-fold) decline in $V_{C_{max}}$ in all genotypes (Figure 4). However, the $V_{C_{max}}$ decline should be more pronounced than was found in our experiment.
Flexas et al. (2006) achieved 94% decrease in the Vc max of Nicotiana tabacum plants resulting from inhibition of Rubisco activity as was also confirmed by other works (Medrano et al., 1997; Parry et al., 2002). Lawlor and Tezara (2009) studied the problem of Rubisco inhibition under drought in more detail and concluded that a key for the response was a decline in the Rubisco activase enzyme activity. Similarly, Lawlor and Cornic (2002) also reported that decreased Rubisco activase activity resulted from progressive water stress.

During our experiment, the dependence between g m and C i was clearly demonstrated (Figure 5). Plants also differed in their g m sensitivity to changing C i. Previously, a rapid response of g m as found in our study has also been reported by Centritto et al. (2003), Flexas et al. (2007, 2014), Bunce et al. (2009), and Keenan et al. (2010), and their mutual dependence was found to be statistically significant ($r^2 = 0.77$). Based on our results, we support the suggestions of Flexas et al. (2006, 2007) and Warren et al. (2008) in that these two parameters of the CO 2 diffusion pathway in photosynthesizing leaves are dependent on each other. This work has also shown that the relationship is highly variable in many species and could be affected by a variety of environmental factors.

Although the increase in stomatal (L S) and mesophyll (L M) limitations to photosynthesis as a result of water scarcity is quite well-documented, processes linked to these phenomena are still a matter of debate (Flexas and Medrano, 2002). Restricted CO 2
diffusion from the surrounding atmosphere to chloroplasts is a common response to water deficit and is caused by limiting factors to photosynthesis even under mild stress conditions (Roupasard et al., 1996; Grassi and Magnani, 2005; Chaves et al., 2009). To study the impact of drought and to demonstrate which limits of photosynthesis dominate, A_{N} - C_{1} response curve analyses are often used (Ni and Pallardy, 2009).

In our analysis of L_{S} and L_{M} under progressive drought stress (Figure 7), genotype differences in these parameters were observed. A variety of differences could be dependent on both the intensity and duration of stress, as well as different abilities to respond to water shortage (Grassi and Magnani, 2005). Under the initial water stress, L_{S} dominated over L_{M} in the Pehlivan genotype (Figure 6). Furthermore, as the water stress developed, L_{M} increased and became crucial. The reason was simply the decline of g_{m}, which was caused by the reduced CO_{2} concentration within chloroplasts. However, L_{S} has not yet been distinguished at this point in comparison with L_{M}. This was caused by only a slight change in the intercellular CO_{2} concentration (C_{i}), as also found by Lawlor and Cornic (2002). Of course, L_{S} increased as well. However, its development was less sufficient compared to L_{M}. The same result for the function of L_{M} was reported in the studies by Galmés et al. (2007) and Tosens et al. (2012).

Our photosynthesis limitation analysis showed that the dynamics of the changes in L_{S} and L_{M} were different in genotypes GK Forrás, Piopi-4 and Šamorínska (Figures 7A,C,D). Since the beginning of dehydration, L_{M} and L_{S} have increased concurrently, as was also observed by Martin-Ruiz and Torres (1992). However, L_{M} began to dominate immediately from the first day of dehydration, as was also observed in the work of Delfine et al. (2001). They argued that the high values of L_{M} indicate the reduction of g_{m} and that the increase in L_{M} is responsible for the impairment of plant metabolism. L_{M} values above 80% were also demonstrated by Galle et al. (2009) in tobacco plants. Other studies (Escalona et al., 1999) observed significant increases in L_{S} and L_{M} at the same time of a stress. Finally, we obtained similar results as documented in the studies of Flexas et al. (2014), Limouzin et al. (2010), Misson et al. (2010), and StPaul et al. (2012), which stated that L_{S} is a more important factor during early drought events; however, under severe water stress, L_{M} dominates over L_{S} and primarily limits wheat photosynthesis.

CONCLUSIONS

The present results show a significant inter-genotypic variability in wheat photosynthetic responses to a long-term progressive drought, as studied in four selected wheat genotypes of different geographical origins and breeding chronology. Our study demonstrated the effect of low water availability in plants on g_{m} inhibition. Drought clearly reduced g_{m} during long-term progressive dehydration in all wheat genotypes. The results show that g_{m} is co-regulated with g_{s}, with their strong effect on A_{S} regulation. Interestingly, g_{m} is a genotypic variable not only for the conditions of drought but also for well-watered plant conditions. Therefore, we offer reliable evidence of a crucial role for g_{m} in the regulation of CO_{2} assimilation under both well-watered and drought conditions. We also demonstrated a rapid response of g_{m} to short-term C_{i} changes with significant genotypic variability under WW conditions. However, this response is significantly reduced without any genotypic effect during prolonged drought. For future research, we suggest the study of leaf anatomical traits linked to the limitations of photosynthesis together with an evaluation of plant photosynthetic parameters. It has been hypothesized, and in some individual works already demonstrated, that the differences in leaf anatomy may have a rather significant influence on the CO_{2} diffusion within the leaf mesophyll and on the whole leaf photosynthetic performance. In summary, the present results with wheat are statistically remarkable, and they contribute to the general knowledge of the regulation of leaf photosynthesis under periods of water scarcity by the mesophyll and stomata.

AUTHOR CONTRIBUTIONS

HS, KO, MB designed the experiment and revised the paper; MK, MZ performed the experiment; KO, PS, MZ, MK analyzed the data and finished the original paper.

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