Effect of drought and heat stress on pollen-pistil interaction, fertilisation and kernel development in wheat

NKFIH-OTKA K108644 (2014-2019)

Final Report

The main objective of the project was the complex characterization of the effect of simultaneous heat and drought (HD) stress from the onset of microgametogenesis to fertilisation in wheat, with emphasis on changes in the morphology and molecular events. The whole phenomenon was studied by an integrated approach comprising cytology, histology, physiology, and proteome and transcriptome analysis.

1. INTRODUCTION

Wheat has a leading role in human nutrition and animal feed in the world with the largest harvested area (220.1 million hectares) and the second largest production (749.5 million tons) among cereals (FAOSTAT, 2016). With the continuously rising human population of the planet and the constant decline in agricultural land availability and quality, forecasted trends of yield increase will not be sufficient to satisfy future demand (Ray et al., 2013; Zandalinas et al., 2018). The enhancement of yield stability even under unfavorable environmental conditions is one of the primary goals of wheat breeders (Lamaoui et al., 2018). Among the extreme weather events, high temperature and drought are expected to be the main yield decreasing factors (IPCC, 2014; Lesk et al., 2016). Globally, drought accounts for 21% yield loss on average (Daryanto et al., 2016). A 1 °C increase in global temperature could reduce the global wheat yield by 4.1% to 6.4% depending on the method used for yield projection (Liu et al., 2016). It has been reported that more than 40% yield fluctuation of wheat can be attributed to climate change (heat waves and drought) at the global, national and subnational scales (Zampieri et al., 2017).

The sensitivity of a plant to environmental factors depends on the species, genotype and developmental stage, and on the duration and severity of the stress. Heat and drought stress during reproductive development may seriously affect crop yields (Barnabás et al., 2008), which can be attributed especially to the high sensitivity to stress shown by pollen development (Jäger et al., 2008; Lalonde et al., 1997; Saini and Aspinall, 1981; Saini et al., 1984; for reviews see Dolferus et al., 2011; De Storme and Geelen, 2014). There are emerging evidences of the sensitivity of female reproductive cell and organ development to heat or drought stress per se in sorghum, rice, maize, wheat, tomato, and canola (Djanaguiraman et al., 2018; Jagadish et al., 2010; Mitchell and Petolino, 1988; Onyemaobi et al., 2017; Pan et al., 2018; Prasad et al., 2011; Polowick and Sawhney, 1988; Saini and Aspinall, 1981; Saini et al., 1983. Majority of studies focus on the effect of heat or drought stress during meiosis and anthesis and little attention has been paid to gametogenesis. During this process, if undisturbed, the sexual organs and gametes complete their development, reach their final size and accumulate the starch reserves needed for successful fertilization and the nourishment of the first cell division cycles of the embryo and the endosperm. Despite their central role in plant reproduction, the vulnerability of wheat pistils to heat or drought stress

has hardly been investigated to date (Saini and Aspinall, 1981, 1982; Saini et al., 1983; Prasad and Djanaguiraman, 2014; Onyemaobi et al., 2017). Although Prasad and Djanaguiraman (2014) found that wheat stigmas and ovaries became desiccated following exposure to high temperature for 5 days before anthesis and that the pollen capturing ability of the stigma decreased, no information was given on the structural changes and processes underlying this phenomenon. However, the stigma, which plays an essential role in reproductive processes, is the most delicate but the least protected female organ, making it the most sensitive to adverse environmental conditions. If receptive, it provides the exact conditions required for pollen germination and the sustained growth and guidance of the pollen tube through the pistil and ovary (Heslop-Harrison, 2000), but no information was available on the effect of HD co-stress on its anatomy and functionality.

Both extreme high temperatures and water shortage lead to the excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which function as signal transduction molecules, but can also cause extensive cellular damage when the balance between the production and scavenging of these compounds is impaired (Hasanuzzaman et al., 2012; Choudhury et al., 2017; Zandalinas et al., 2018). ROS and RNS are partially reduced or activated forms of molecular oxygen and nitrogen (del Río, 2015). Small amounts of these radicals and compounds are produced continuously even under favorable conditions, particularly in the plastids, mitochondria, peroxisomes, cytosol and apoplast. The most important types of reactive radicals and compounds are singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), nitric oxide (NO) and peroxynitrite ($ONOO^-$; Molassiotis and Fotopoulos, 2011; Demidchik, 2015). These molecules differ greatly in their lifespan, on a nanoseconds to seconds scale. ROS and RNS also show diverse reactivity, from moderate ($O_2^{\bullet-}$) to very high ($OH^{\bullet}$, $ONOO^-$; Waszczak et al., 2018), being able to oxidize lipids, proteins, carbohydrates and nucleic acids, therefore effectively impairing the structural integrity of cells when present in large amounts (Vandelle and Delledonne, 2011; Demidchik, 2015). On the other hand, the signaling role of ROS and RNS has been revealed in both developmental and stress reaction processes in the past decade (Waszczak et al., 2018). Although a certain amount of information is available on the role of the ROS content of the stigma and stigmatic papillae in developmental changes and pollen incompatibility processes (McInnis et al., 2006; Serrano et al., 2010; Domingos et al., 2015; Zafra et al., 2016), there are no data on the environmental stress-induced ROS and RNS generation in this delicate and important organ. As generative processes show significant vulnerability to heat and drought stress, it can be hypothesized that ROS and RNS play an important role in the reduction in fertility and in consequent yield loss.

High temperature and drought often occur simultaneously during plant development causing severe yield loss in most wheat-growing areas. The sensitivity of Angiosperms to simultaneous heat and drought stress is not well understood (Pradhan, 2012). The growth, physiological and metabolic responses of plants to a combination of heat and drought (HD) stresses are unique and cannot be directly extrapolated from the responses to each of these stresses separately (Rizhsky et al., 2002, 2004; for reviews see Barnabás et al., 2008; Dolferus et al., 2011; De Storme and Geelen, 2014). Different stress combinations should be handled as a new state of stress in plants, requiring novel types of defense and acclimation responses (Suzuki et al., 2014).

Addressing the morphological, anatomical, physiological and molecular mechanisms conferring sensitivity and tolerance of wheat to HD co-stress will help to develop genotypes

capable of adapting to a changing climate. Hence the objectives of this project were to (1) reveal the effect of heat and drought stress during gametogenesis on the morphology, structure and functionality of vegetative organs and yield components of wheat genotypes with contrasting HD tolerance and (2) on the morphology, structure and functionality of reproductive cells and organs; (3) shed light on HD stress induced ROS and RNS generation in stigmatic papilla cells; (4) unravel the link between HD stress-induced oxidative damage and fertility loss; (5) identify HD induced changes in stigma proteome and (6) transcriptome.

2. RESULTS AND DISCUSSION

2.1 HD co-stress reduced water content of the plants, increased canopy temperature and accelerated vapor pressure deficit

However, the volumetric water content (VWC) of the soil dropped uniformly below 7% in both genotypes as a consequence of combined heat and drought stress applied for 5 days prior to anthesis, there was a significant ($P \leq 0.005$) difference in the reduction of the relative water content (RWC) of the genotypes. The reduction in Cappelle Desprez RWC (control: 87.6%, HD-stressed: 50.1%) was significantly ($P \leq 0.05$) more pronounced than in Plainsman V (control: 88.6%, HD-stressed: 70%). The flag leaves of treated Cappelle Desprez plants showed visible

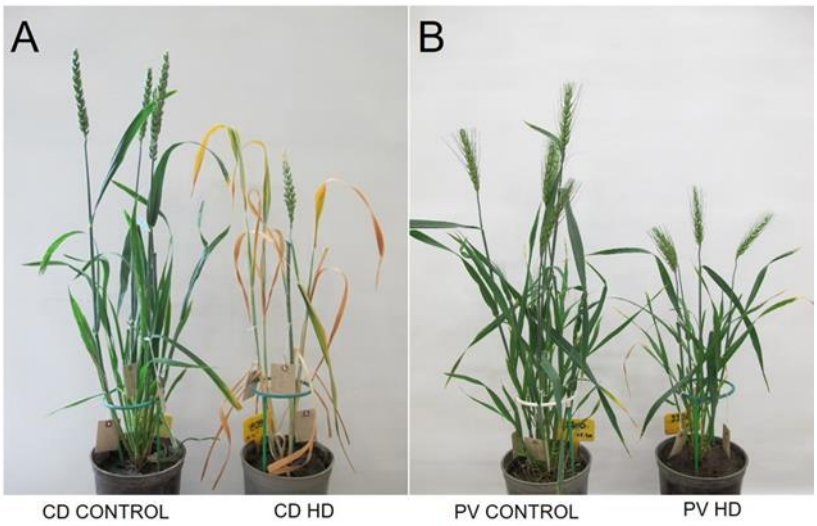

Figure 1. Control and HD stressed plants of the sensitive Cappelle Desprez (A) and tolerant Plainsman V (B) winter wheat varieties 5 days after anthesis. Note that DH strongly reduced the plant height in both genotypes. In contrast to the tolerant variety, vegetative organs of treated Cappelle Desprez plants were severelly damaged and could only partially regenerate.

symptoms of dehydration, starting around noon on the 3rd *day* of treatment, while leaf rolling was only observed on the flag leaves of treated Plainsman V plants on the 5th day of treatment. Whereas plant of the Plainsman V genotype could recover after re-irrigation completely, the canopy of Cappelle Desprez suffered severe damage (Fig. 1). There was a significant ($P \leq 0.05$) difference between the relative water losses (RWL) of the genotypes. In contrast to Plainsman V, flag leaves of the sensitive genotype lost water content on a significantly higher extent that indicates greater residual transpiration thus, the presence of a thinner cuticule in accordance with our previous results (Jäger et al., 2014). As a consequence of HD the ΔT dropped significantly by 53% and 35% in Cappelle Desprez and Plainsman V, respectively. The midday vapor pressure deficit increased by 529% and 387% in plants of the sensitive and tolerant variety, respectively.

HD co-stress altered fluorescence ratio parameters and photosynthesis

To assess HD induced photochemical changes of PSII complexes, fluorescence ratio parameters: maximum photochemical quantum yield (F_v/F_m), quantum yield of photochemical

energy conversion in PSII (ΦPSII), photochemical quenching parameter related to the fraction of open reaction centres in PSII (qP) and non-photochemical quenching parameter (NPQ) of control and treated plants were determined and compared. Neither species, nor developmental stage dependence was observed in the fluorescence ratio parameters of control plants. The average Fv/Fm, ΦPSII, qP and NPQ values of control wheat plants were 0.80, 0.66, 0.88 and 0.38, respectively. HD stress triggered significant ($P \leq 0.005$) decline of Fv/Fm, ΦPSII and qP parameters in both genotypes to the end of HD treatment. Compared to the reduction of Fv/Fm in Plainsman V (7%), the drop of Cappelle Desprez (42%) was more pronounced. The Φ_{PSII} was reduced by 63% and 11% in Cappelle Desprez and Plainsman V genotypes, respectively. Compared to their respective controls, the proportion of open PSII reaction centres was much lower in Cappelle Desprez (60%) than in Plainsman V (95%). A sharp, 40% decrease in NPQ was observed in treated leaves of Cappelle Desprez. The general trend in the response of net photosynthesis (P_n), stomatal conductance (g_s), transpiration rate (E) and instantaneous carboxylation efficiency (P_n/Ci) to HD co-stress, and the magnitude of changes was similar in both cultivars (Fig. 2A-E). Compared with their

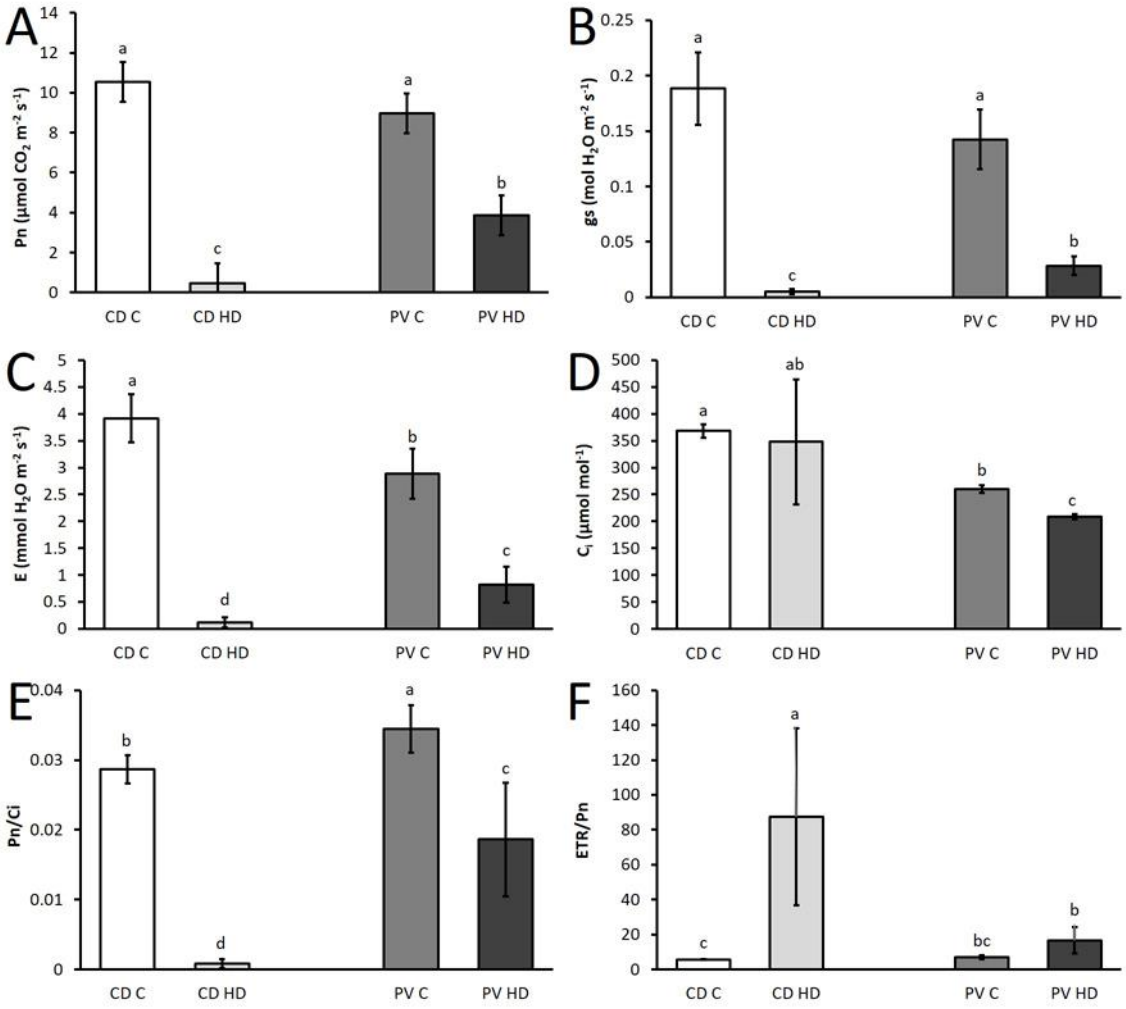

Figure 2. Net photosynthesis (A), stomatal conductance (B), transpiration rate (C), intracellular CO_2 concentration (D, instantaneous carboxylation efficiency (F) and the ratio between electron transport rate and net photosynthetic rate (F) of Cappelle Desprez and Plainsman V wheat genotypes under optimum and HD co-stress conditions. CD: Cappelle Desprez; HD heat and drought co-stress; PV: Plainsman V. Columns represent means ± standard deviations; different letters in each graph indicate statistically significant differences at P ≤ 0.005 level of probability.

controls, the reduction of P_n, g_s, E and P_n/C_i at anthesis (end of treatment) was 96%, 97%, 97% and 97% in Cappelle Desprez and, 56%, 80%, 72% and 45% in Plainsman V (Fig. 2A,B,C,E), respectively. HD co-stress triggered significant ($P \leq 0.05$) reduction of C_i exclusively in the flag leaves of Plainsman V (20%) at the end of treatment that indicates that only the tolerant genotype could maintain photosynthesis even under severe co-stress conditions (Fig.2D). The increased ETR/P_n ratio of Cappelle Desprez (from 6 to 87; Fig. 2F) indicates that excessive energy was dissipated in the system. HD co-stress had no effect on the ETR/P_n ratio of Plainsman V.

Degradation of proteins and photosynthetic pigments

On a dry weight basis (g), the protein content of control Cappelle Desprez and Plainsman V flag leaves was 617 mg and 682 mg, respectively. Simultaneous HD co-stress induced significant ($P \leq 0.005$) decrease in protein content of both genotypes. Compared to the remarkable protein loss in Cappelle Desprez (78%), reduction in Plainsman V (53%) was rather moderate. HD co-stress induced significant ($P \leq 0.005$) loss of photosynthetic pigments. Trends of the changes were similar for both cultivars, but the extent of reduction in chlorophyll a, b and carotenoid contents varied with the genotype. On a dry weight basis, HD co-stress reduced the amount of chlorophyll a and b by 77% and 79%, respectively, in Cappelle Desprez, and by 43% and 42%, respectively, in Plainsman V. Greater reduction in photosynthetic pigments occurred in Cappelle Desprez, implying that a slower degradation of chlorophyll for Plainsman V enabled better light absorption capacity. Significant increase of the chlorophyll a/b ratio detected in Cappelle Desprez (14%), indicated that compared to Plainsman V, HD stress reduced the chlorophyll b content in the flag leaves on a higher rate. The carotenoid content decreased in parallel with chlorophyll a + b, however, to a lower extent. Stress-induced reduction of carotenoids was 69% and 36% in Cappelle Desprez and Plainsman V, respectively. The carotenoid-to-chlorophyll ratio decreased significantly with stress in both genotypes, however, compared to the 9% reduction in the tolerant cultivar, the 29% reduction in Cappelle Desprez was more pronounced.

Reduced antioxidant enzyme activities

Under control conditions no significant ($P \leq 0.005$) genotype-dependent differences were observed in the activities of various antioxidant enzymes (Fig. 3). The overall activity was reduced in HD stressed flag leaves of both cultivars, however the decline was more pronounced in the Cappelle Desprez variety. The flag leaves of the HD co-stressed sensitive genotype encountered a massive 85% loss in catalase activity, which was 39% lower than that of Plainsman V (Fig. 3A). The guaiacol peroxidase activity was reduced by 71% and 42% in Cappelle Desprez and Plainsman V, respectively (Fig. 3B). Decline in ascorbate peroxidase activity showed similar characteristics, the HD co-stress induced activity in the sensitive variety was 31% lower than that of the tolerant one (Fig. 3C). As a consequence of treatment, glutathione reductase activity was reduced by 70% and 46% in Cappelle Desprez and Plainsman V, respectively (Fig. 3D). The values for glutathione-S-transferase reduction in the sensitive and tolerant genotype were 77% and 42%, respectively (Fig. 3E). There was no difference between the relative antioxidant capacity of the genotypes under control conditions, however, as a consequence of HD co-stress, the indices were severely reduced to

the end of treatment in both genotypes. In Cappelle Desprez and Plainsman V the RAC dropped from 4.01 to -6.12 and from 4.00 to -1.90, respectively (Fig. 3F).

The level of lipid peroxidation was determined through the estimation of malondialdehyde (MDA) equivalents content. A sharp increase in MDA content was observed under HD stress. Stress induced significant oxidation of membrane lipids in both varieties as indicated by 74% and 52% increase in MDA contents of Cappelle Desprez and Plainsman V flag leaves, respectively.

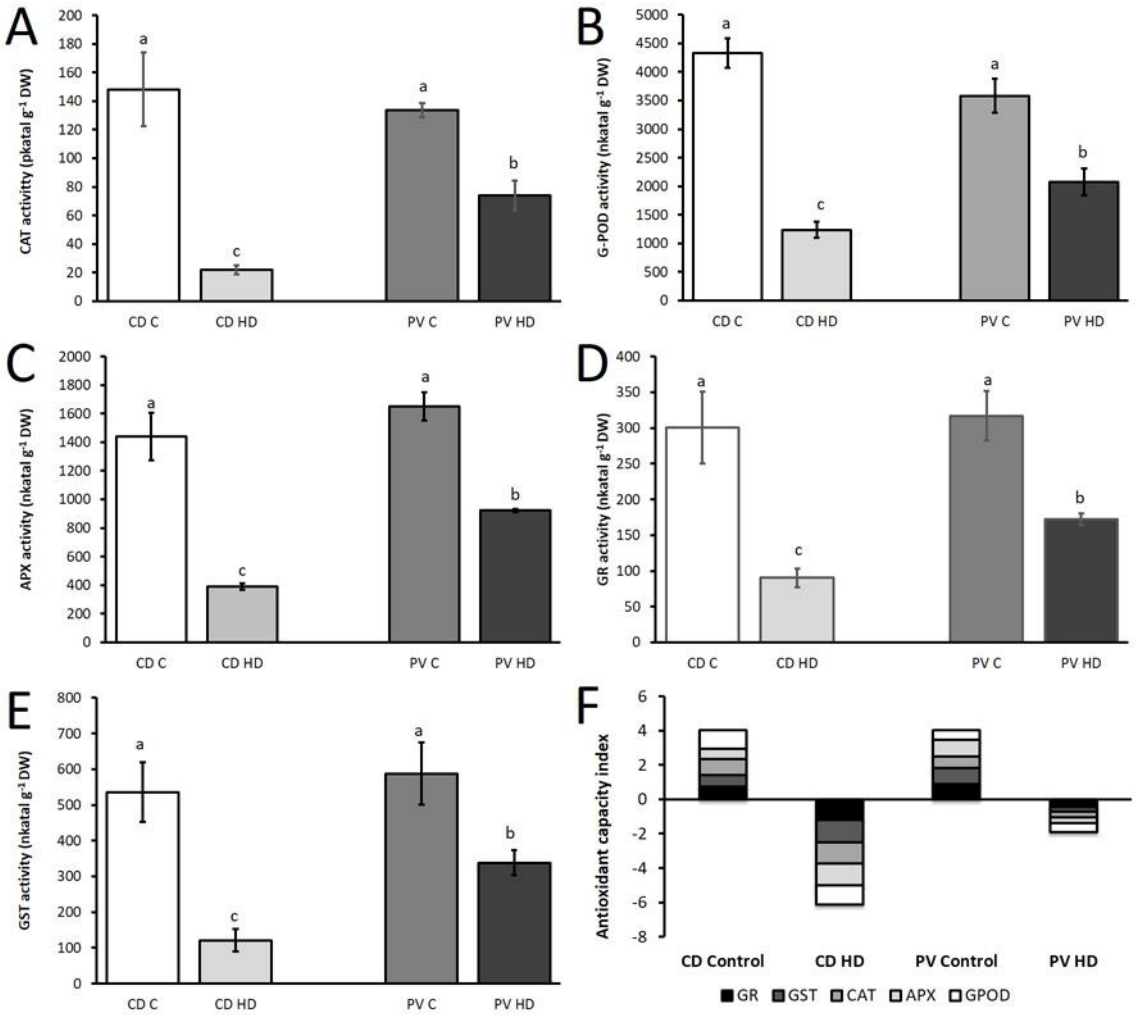

Figure 3. *Activities of catalase (A), guaiacol peroxiadase (B), ascorbate peroxidase (C), gluthation reductase (D), glutathione-S-transferase (E) enzymes, and the antioxidant capacity index of Cappelle Desprez and Plainsman V wheat genotypes grown under control and HD co-stress conditions. APX: ascorbate peroxidase; CAT: catalase; CD: Cappelle Desprez; G-POD: guaiacol peroxiadase; GR: gluthation reductase; GST: glutathione-S-transferase; HD heat and drought co-stress; PV: Plainsman V. Columns represent means ± standard deviations; different letters in each graph indicate statistically significant differences at P ≤ 0.005 level of probability.*

Correlation between photosynthetic parameters and the relative antioxidant capacity (RAC)

Very strong positive correlation (0.869-0.989) was found between, photosynthetic parameters (P_n, E), ΔT, fluorescence ratio parameters (F_v/F_m, ΦPSII, qP, NPQ; Fig. 7B), chlorophyll content, RWC and the RAC. Strong positive correlation (0.760) was found between stomatal conductance (G_s) and the RAC. Very strong negative correlation (0.996) was found between MDA and the RAC of wheat plants.

Changes in phenology due to combined stress

The adverse environmental conditions significantly shortened the duration of gametogenesis, which lasted for 7 days under optimum conditions. Independently of the genotype, HD-stressed plants started flowering 3 days earlier. Moreover, HD stress shortened the duration of grain filling in both Plainsman V and Cappelle Desprez, by 10 and 14 days, respectively.

Simultaneous heat and drought reduced fertility and production

The analysis of yield components revealed that Cappelle Desprez suffered significantly greater loss in plant production after treatment, so this variety was considered as sensitive to HD co-stress. The main spikes of the Cappelle Desprez variety were significantly ($P \leq 0.05$) longer (10.7 ± 0.1 cm) than those of Plainsman V (9.2 ± 0.4 cm), and consisted of 25.4 ± 1.7 and 17.9 ± 1.2 spikelets, respectively. No significant HD-stress dependent reduction in spike length was observed in either of the genotypes. The data indicate that the stress-induced damage to stigmatic papilla cells in Cappelle Desprez, which was verified by the results of anatomical observations in the present project, strongly contributed to the decrease in function and fertility. Spike fertility, which determines grain number and sink strength, is a crucial factor in wheat yield potential (Reynolds et al., 2009). Heat and drought stress significantly decrease fertility (Prasad et al., 2011), which is generally considered to be the consequence of the damage sustained by the male gametophyte, while the role of pistil dysfunction in yield loss is somewhat underestimated. The loss observed in the fertility of the HD-stressed sensitive genotype in this study highlighted the fact that not only the extensively studied meiotic processes, but also development of sexual organs taking place during gametogenesis is highly sensitive to a changing climate. Apart from the great sensitivity shown by pollen development to heat and drought stress (Jäger et al., 2008; Lalonde et al., 1997; Saini and Aspinall, 1981; Saini et al., 1984; for reviews see Dolferus et al., 2011; De Storme and Geelen 2014), the results of the pollination experiment demonstrated that the damage to female reproductive organs induced by HD stress reduced their functionality and was responsible for 34% of gross fertility loss. Irrespective of the genotype and treatment, the grain number and fertility of florets developing in the upper part of the spikes were significantly lower than the relevant parameters of the basal florets. It might be expected that decreased fertility would be negatively correlated with TSW due to compensation. This was not true of Plainsman V, in which neither fertility nor TGW was influenced by the treatment. In contrast, the fertility of Cappelle Desprez dropped significantly by 39% and 56% in the basal and top floret positions, respectively, as a consequence of HD which indicates that this variety was unable to compensate for the reduced grain number by an increase in grain weight. Moreover, although the duration of grain fill was shortened by 10 and 14 days in the tolerant and sensitive genotype, respectively, the former gave a plant production only 8% less than the control, while the production of the latter dropped in both spike halves by 55% on average. Prasad and co-workers (2011) reported a similar decrease in the number of days to physiological maturity of bread wheat cultivars under a combination of drought and high temperature stress applied at heading.

2.2. Floret position and HD-triggered stigma dysfunction influenced fertility

In order to assess the effect of the stigma dysfunction triggered by HD stress on fertility loss in the sensitive genotype, a pollination experiment was conducted on Cappelle Desprez plants, where the effect of floret position, floret truncation and manipulation was also considered. Floret location (top or base of the spike) had a significant effect on fertility in both control and

HD-stressed Cappelle Desprez plants. The cutting off one-third of the bracts (glumes, paleas, lemmas) in the primary florets and the removal of the central florets (truncation, group iii) from the control spikelets caused a position-independent loss in fertility (base: 21%, top: 25%). Compared to the truncated and free-pollinated control florets (group iii), the hand pollination of truncated and emasculated (manipulated, group iv) control florets with control pollen did not reduce fertility. Compared to the free-pollinated intact control florets (group ii), HD stress induced a significant 62% and 33% loss in the fertility of truncated free-pollinated (group iii) and manipulated and hand-pollinated (group iv) florets, respectively (Figure 4). The removal of one-third of the bracts had a similar fertility-reducing effect in HD-treated florets (base: 24%; top: 26%) as in the control. In free-pollinated HD-stressed spikes, there was a significant difference between the fertility loss in florets located in the base (45%) and top (56%). Both pistil- and-pollen dependent fertility loss varied significantly with position, being 22% and 36% in florets located in the upper halves of the spikes and 15% and 32% in basal florets, respectively. The competition for assimilates between the upper and basal spikelets is a well-known phenomenon that could be more exacerbated when the plants encounter drought stress (Chen et al., 2013), high temperature stress (Fu et al., 2016) and their combination.

Effect of co-stress on spike and anther morphology, pollen viability and pollen germination

Compared to their respective controls, there was no change in spike morphology in Plainsman V a week after HD treatment, while the bracts of the apical florets in treated Cappelle Desprez spikes turned yellow and the spikes showed strongly reduced fertility. No reduction in anther length was observed after HD stress. The mean viability of Plainsman V pollen (80.4% ± 2.7) was independent of the treatment or floret position. In contrast, for the genotype Cappelle Desprez, lower pollen viability was observed in superior spikelets than inferiors under both optimum conditions and heat and drought co-stress. Compared to the base (74.6% ± 4.3) the viability of control pollen located in the top anthers was significantly (26%) lower. In agreement with reports of the pollen viability-reducing effect of high temperature or drought (De Storme and Geelen, 2014). HD stress severely reduced pollen viability by 63% and 81%, in the basal and top halves, respectively, of treated spikes of the sensitive genotype. In contrast to viability measurements, control Cappelle Desprez pollen showed higher germination frequency (48%) than Plainsman V. However, compared to Plainsman V pollen (37%), depression in the germination ability of Cappelle Desprez was much higher (94%), and only 2% of the pollen cells was able to form a pollen tube.

Morphological and anatomical changes induced by HD stress in pistils

As in many members of the *Poaceae* family, the stigma of wheat is dry and plumose and the pistil is bifurcated. The primary branches, known as stylodia, are densely covered with multiseriate secondary branches consisting of papilla cells. As a consequence of HD co-stress, the length of the stylodia was significantly reduced in both genotypes probably as a consequence of the arrested cell enlargement induced by a significant decrease in plant RWC. This contrasts with the findings of Jagadish and co-workers (2010) and Pan and co-workers (2018), who reported unaffected stigma length and stigma exsertion, respectively, after high temperature stress. The extent of genotype-independent reduction varied with floret position, averaging 35% at the base, 12% in the center and 31% at the top of the spikes. Some of the HD stressed Cappelle Desprez stylodia were shriveled, with fewer secondary branches (Figure 4). While no signs of dehydration or anatomical anomalies were observed in the pistils of the tolerant genotype, moderately dehydrated secondary stigmatic branches were typical

of HD-stressed Cappelle Desprez pistils isolated from the upper half of the spikes. A similar phenomenon was observed in wheat and sorghum stigmas when exposed to heat stress per se (Djanaguiraman et al., 2018; Prasad and Djanaguiraman, 2014). Light microscopic studies using differential interference contrast and Syto 63 staining revealed the structural effects of HD treatment on the stigmatic papillae. Untreated stigmatic papilla cells possessed disk-shaped nuclei wedged tightly in between the large vacuoles that occupied almost the whole of the cell. The cytoplasm was generally located on the periphery of the papilla cells, although large cytoplasmic segment was also found in control outwardly curved apical papilla cells. The stigmatic papilla cells of Cappelle Desprez pistils, especially those located in the top half of the spikes, showed signs of injury after treatment. The nuclei were reshaped and relocated from their original position. Syto 63 staining revealed fragmentation of the nuclei and cytoplasm.

The transversally cut surface of both control and HD-stressed stylodia was somewhat ovate at a quarter of the way from the top, and consisted of large, vacuolated cortical cells surrounding a few small, cytoplasm-rich transmitting cells. Although HD stress had no effect on the structure of Plainsman V stylodia, whether isolated from the top or base of the spike, those in the upper part of Cappelle Desprez spikes were slightly dehydrated. Halfway down the spike, control stylodia and those of HD-stressed Plainsman V had a shield-like shape. A massive vascular bundle surrounded by cortical cells was located on the lateral side of the stylodia, the medial side consisted of small, turgid, well-demarcated transmitting cells with round nuclei. Multiple layers of large, vacuolated cortical cells connected the two sides of the stylodium. By contrast, as a consequence of the HD stress-induced extensive degeneration of almost all the cortical cells and part of the transmitting tissue, a mass of crushed cells was visible in HD-stressed stylodia isolated from the top half of the sensitive genotype, and as a consequence of the damage these turned into dumbbell-shaped bilobed structures. A few intact cortical cells were visible exclusively in the proximity of the vascular bundle. No such environmental stress-induced structural anomalies have been described in Angiosperms so far.

Irrespective of the genotype and treatment, unilocular ovaries and ovules showed similar anatomy in both control and HD-treated florets. No signs of dehydration were observed in these well protected organs. The effect of water withdrawal on wheat ovule development is rather controversial. As found by our team, Saini and Aspinall (1982) reported that water withdrawal per se had no effect on ovule development. In contrast, Onyemaobi and co-workers (2017) considered that female reproductive organs could be one of the major contributors to low seed set in wheat stressed during meiosis. It is important to note that the male and female gametophytic processes in *Triticum aestivum* are not synchronized after meiosis and that the differentiation of the octonucleate embryo sac (female gametophyte) proceeds far more rapidly than that of its male counterpart (Tímár et al., 1997). It can be assumed that the 7-celled female gametophyte was already formed by the time the microspores entered the binucleate stage of development, which means that the development of the female gametophyte was accomplished by the 2nd day of HD stress, which is why the treatment had no negative effect on the structure of the ovules. The style, protruded into the ovary consisted of 10-15 rows of vacuolated spindle-like cells with large ovate nuclei. At the base the style took the form of a funnel that ended between the tube cell layer and the outer layer of the outer integument. The ovaries were well developed; the mesocarp cells accumulated a large amount of starch. The cross and tube cell layers were discontinuous at the base of the two styles, where the cells of the style were loosely connected to the outer layer of the outer integument. Chloroplasts were visible in the dense cytoplasm

of both the cross and tube cell layers, but chloroplasts accumulating starch deposits were only visible in a few cell rows lining the base of the style. The ovary contained a single embryo sac surrounded by integuments. The double layer of the outer integument was highly vacuolated. The two layers of the inner integument formed the micropyle at the base of the ovule. The ovules were lined with the nucellar epidermis and nucellus. However, none of these were present in the vicinity of the micropyle, while large quantities of nucellar cells were observable at the chalaza. Irrespective of the treatment, degraded nucellar cells were found in the proximity of vacuolated antipodal cells. The egg apparatus consisted of two highly vacuolated synergids with well-developed filiform apparatus, and the egg cell. Regardless of whether the two polar nuclei of the central cell were adjacent to the egg cell or not, they were attached to it by means of cytoplasmic bridges. Both the egg cell and the central cell accumulated starch. Our results are in contrast to the findings of Saini and co-workers (1983), who reported that the embryo sacs were completely absent or formed abnormally with incomplete cellular organization and altered ovary development (reduced nucellus development, over-proliferated integuments) in pistils subjected to continuous exposure to 30 °C for 3 days during meiosis.

HD altered pollen development

When semi- and ultrathin sections of the pollen cells were examined, the HD co-stress was found to have no effect on the morphology of Plainsman V pollen. The pollen isolated from the upper third of treated spikes of Cappelle Desprez exhibited a considerable loss of water (Fig. 4). Compared to the control pollen, the intine was significantly thicker in these cells, less starch could be observed in the cytoplasm and the mitochondria were far larger (Fig. 4F). This difference in size (indicating the loosening of the cell organelle structure) was probably the consequence of the reduction or termination of outer and inner membrane integrity. No genotype or treatment dependent difference was observed in the structure of the anthers what indicates that the tapetum showed symptoms of senescence at anthesis.

HD co-stress triggered size reduction and anatomical anomalies of both flag leaves and mesophyll chloroplasts

The flag leaves of control Plainsman V plants were significantly ($P \leq 0.005$) smaller than those of the sensitive cultivar (Table 1). Although combined heat and drought stress had no effect on the expansion of Plainsman V flag leaves, both the length and the area of Cappelle Desprez flag leaves were significantly ($P \leq 0.05$) reduced by 19% and 28%, respectively (Table 1), as a consequence of stress treatment. There was a genotype-dependent difference observed in the size of mesophyll chloroplasts. The area of the chloroplasts in control Plainsman V flag

Table 1. Flag leaf area and flag leaf length of control and HD co-stressed Cappelle Desprez and Plainsman V plants.

	CD C	CD HD	PV C	PV HD
Flag leaf length (cm)	33.47 ± 0.93^{a}	27.41 ± 1.03^{b}	27.64 ± 1.01^{b}	29.52 ± 1.48^{b}
Flag leaf area (cm^2)	52.43 ± 4.18^{a}	37.16 ± 4.94^{b}	31.20 ± 1.55^{b}	34.07 ± 1.17^{b}

Values represent means ± standard deviations of three independent biological repetitions. In each row, means superscripted by different letters are significantly different at the $P \leq 0.05$ level of probability. C: control; CD: Cappelle Desprez; HD: heat-and-drought stress; PV: Plainsman V.

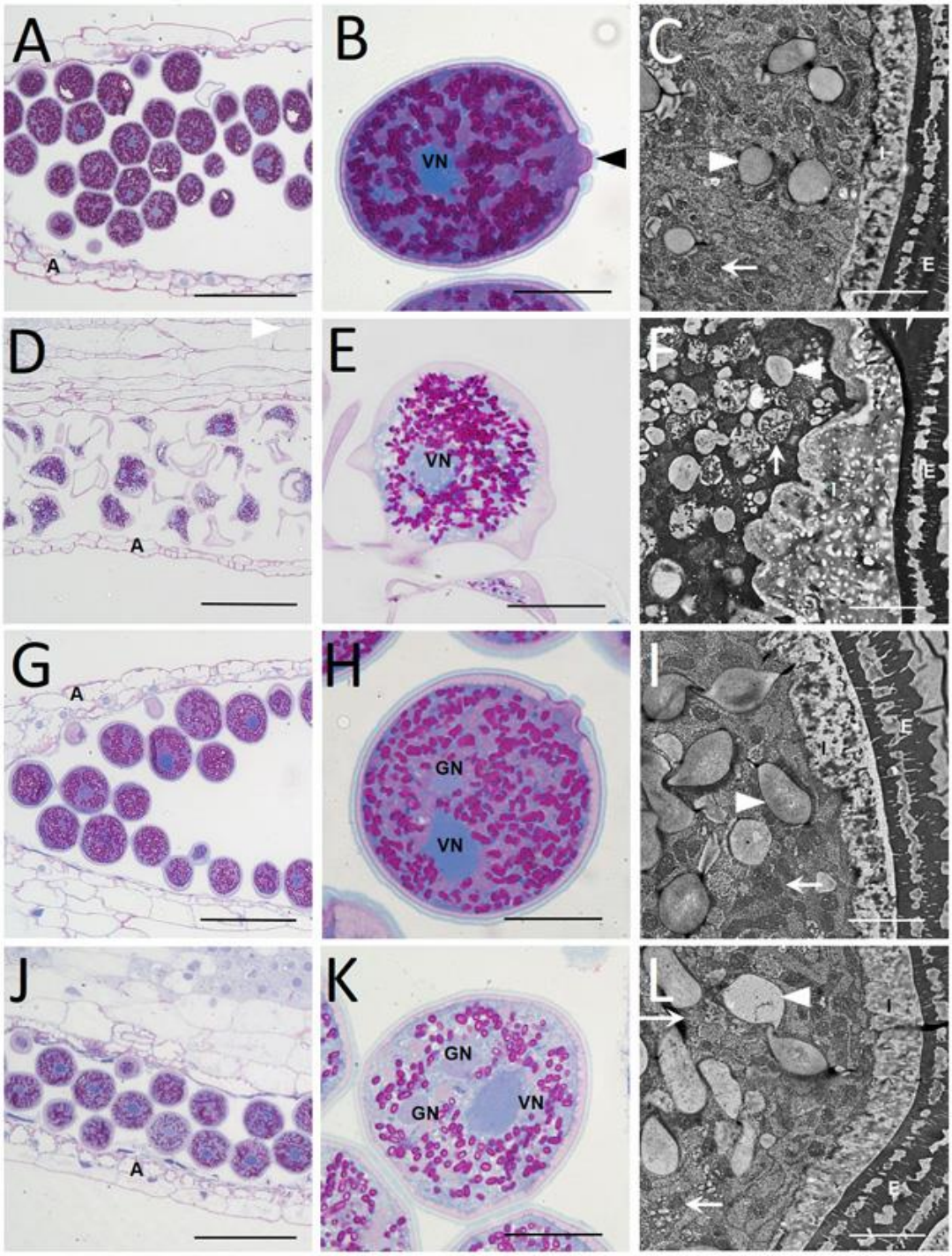

Figure 4. *Morphology and ultrastructure of control (A-F) and HD co-stressed (G-L) wheat pollen cells. Under control conditions anthers of both Cappelle Desprez (A) and Plainsman V (G) contained spherical trinucleted pollen cells with large starch reserves (B,H) and native ultrastructure (C,I) at the onset of anthesis. As a consequence of HD co-stress in Cappelle Desprez anthers (D), shrivelled pollen with reduced starch reserves (E) and, on ultrastructural level, swallen mitochondria with broken cristae and low matrix density, smaller starch granules and thick, disorganized intine were observed (F). In contrast, compared to the control (H), the pollen grains of treated Plainsman V deposited less starch (K), did not show signs of plasmolysis or mitochondrial damage (L). A: anther; E: exine; GN: generative nucleus; HD: heat and drought; I: intine; VN: vegetative nucleus; arrow: mitochondrion; black arrowhead: starch granule; white arrowhead: operculum; red colour: carbohydrates; blue colour: proteins. Scale bars represent 100 um (A,D,G,J), 20 um (B,E,H,K), 2 um (C,F,I,L).*

leaves was significantly ($P \leq 0.005$) larger (21.0 ± 1.9 μm^2) than in that in Cappelle Desprez (17.2 ± 0.9 μm^2) leaves. Compared to their respective controls, the area of HD stressed chloroplasts was reduced in both varieties, by 16% in Plainsman V (17.6 ± 0.8 μm^2) and by 24% in Cappelle Desprez.

Light and transmission electron microscopy (TEM) was used to shed light on the effect of HD on the micromorphology of flag leaves, both on tissue and sub-cellular level. At the time of flowering, chloroplasts in control mesophyll cells were located evenly around the vacuoles in the vicinity of the plasma membrane, with well observable starch granules in the middle of the organelles (Fig. 5A,B,G,H). The ultramicrographs showed that control chloroplasts possessed clearly defined envelope membranes and compact structure with well-defined stromal thylakoids and tightly packed granas (Fig. 5C,I). Plastoglobules, which are plastid localized lipoprotein particles, were present in control chloroplasts of both genotypes. Neither of the genotypes deposited starch in HD stressed chloroplasts (5D,E,J,K). Comparative analysis of stress-induced structural and ultrastructural changes showed obvious genotype dependency at anthesis (end of treatment). HD stress induced alterations in Cappelle Desprez cells were more prominent. As a consequence of advanced dehydration, bulliform cells located in the upper epidermis of Cappelle Desprez leaves lost their turgor (Fig. 5D). Moreover, no vacuoles were observed in HD treated mesophyll cells of the sensitive variety, thus the chloroplasts were located in the middle of the cells. In contrast to Plainsman V mesophyll cells (Fig. 5K), disintegration was visible in many regions of Cappelle Desprez leaves (Fig. 5E). The outer envelope membranes of Cappelle Desprez chloroplasts in those regions were discontinuous or absent, and the stromal and intergranal lamellar system was distorted with irregular arrangement and random orientation of inflated thylakoids (Fig. 5F). In contrast to Cappelle Desprez (Fig. 5D), no signs of motor cell dehydration were visible in treated Plainsman V flag leaves (Fig. 5J). TEM observations revealed that chloroplasts in mesophyll cells of the tolerant variety were surrounded with intact outer envelopes and the thylakoids were organized into well-defined, in some extent loosely stacked granas (Fig.5L).

HD co-stress had no effect on kernel development

The light microscopic analysis of 3- and 7-day-old wheat caryopses revealed that the negative effect of HD co-stress was not manifested either in embryo or endosperm development. Irrespective of treatment, globular stage embryos with 6 cells visible at the largest embryo area on sections cut at the median plane were observed in 3-day-old caryopses. All embryonic cells contained starch granules. At this stage, free nuclei of the syncytium were evenly distributed in the periphery of the elongated cavity of the ovule. Cell walls were observable exclusively around endosperm cells that were located in the vicinity of the embryo. The antipotal cells were persistent at the chalazal region of the ovule. The non-differentiated globular embryos consisted of approximately 40 cells visible at the largest embryo area in 7-day-old caryopses. The 6-7 row thick cellular endosperm was lined with a single layer of a nucellar endosperm and only the inner cell layers contained small, A-type starch granules. Compared to control 14 day-old caryopses, no stress induced changes in embryo development or starch accummulation in the endosperm were observed either in HD co-stressed Cappelle Desprez or Plainsman V caryopses. It can be assumed that HD co-stress applied from the onset of microgametogenesis to anthesis had no effect on further kernel development and even the partially regenerated sensitive variety could supply (produce or remobilize) assimilates to the reduced number of kernels.

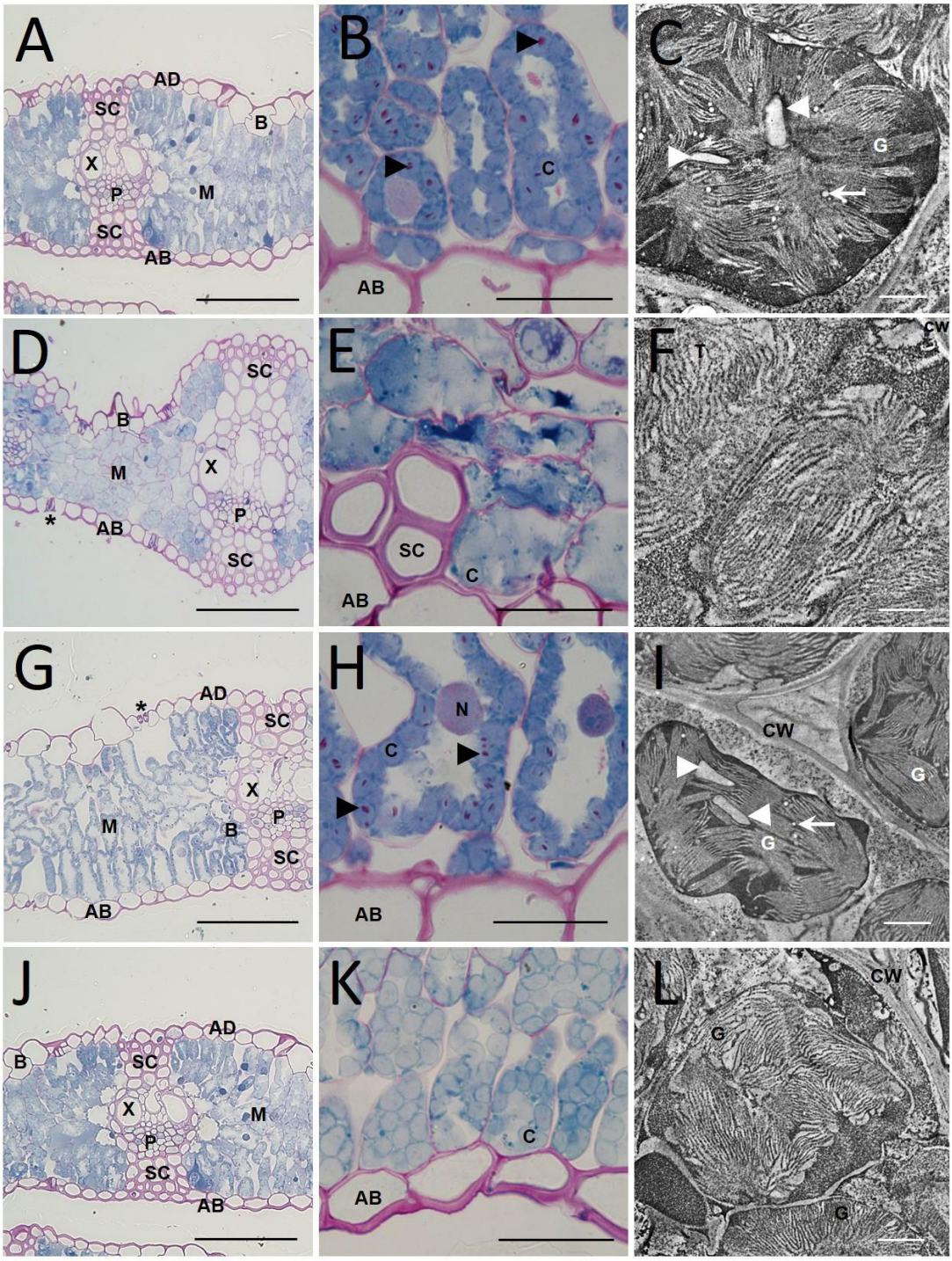

Figure 5. Anatomy of wheat flag leaves grown under control and HD co-stress conditions. Light (A , B, D, E, G, H, J, K) and electron micrographs (C, F, I, L) of the tolerant Plainsman V (A-F) and sensitive Cappelle Desprez (G-L) leaf sheaths (A,D,G,J), mesophyll cells (B, E, H, K) and chloroplasts (C,F,I,L) grown under control (A-C, G-I) and HD co-stress conditions. Note the appressed grana stacks in C, I and L. Chloroplasts in the mesophyll cells of the HeD treated Cappelle Desprez leaves were disintegrated (E) and the granas of the chloroplasts were dissociated (F). AB abaxial epidermis; AD adaxial epidermis; B bulliform cell; C chloroplast; CW: cell wall; G: granum; M: mesophyll cell; N: nucleus; P: phloem; S: stomata; SC: sclerenchyma; T: thylakoid; X: xylem; arrow: plastoglobule; arrowhead: starch granule; asterisk: stomata; red colour: carbohydrates; blue colour: proteins. Scale bars (A,D,G,J) 100 um; (C,E,H,K) 20 um; (C,F,I,L) 1 um.

2.3. Influence of HD on ROS and RNS generation of the stigma

As oxidative stress is an important source of damage when plants face high temperature and water shortage simultaneously (Zandalinas et al., 2018), it can be assumed that ROS-mediated injury is a major factor contributing to structural changes. It was demonstrated here, that structural anomalies triggered by the HD-induced generation of ROS and RNS may stand in the behind of reduced stigma function and female-dependent fertility loss. In photosynthetic tissues, the main sources of ROS are the chloroplast and the peroxisome, especially when conditions are unfavorable (Waszczak et al., 2018). However, as photosynthesis does not occur in stigmatic papilla cells, major ROS-generating processes such as the reduction of O_2 in photosystem I or photorespiration in the peroxisomes are absent. The potential ROS-generating regions in papilla cells are therefore the mitochondria, the glyoxysomes and the plasma membrane-cell wall-apoplast system (Noctor et al., 2014; Singh et al., 2016; Waszczak et al., 2018). Fluorescent ROS indicators provide powerful tools for the investigation of oxidative stress in living cells. Nevertheless, the majority of these probes have limitations due to their insufficient ROS specificity, which should be taken into account when interpreting the results (Ortega-Villasante et al., 2016). The present study confirmed that the stigmatic papillae of monocotyledonous wheat generate high amounts of ROS ($O_2^{\bullet-}$, OH^-, H_2O_2) and RNS ($ONOO^-$, NO) at anthesis. These results are in accordance with the findings of McInnis et al. (2006), Serrano et al. (2010) and Zafra et al. (2016) who observed the accumulation of ROS/H_2O_2 in dicotyledonous angiosperms. In order to estimate the general oxidative stress manifested in the cells, non-fluorescent H_2DCFDA, an indicator of total ROS was added to control and HD-stressed stigmas. H_2DCFDA, after cleavage by oxidation was converted to the highly fluorescent 2',7'-dichlorofluorescein. The fluorescence of the oxidized H_2DCFDA showed vacuolar localization. Irrespective of the genotype a weak fluorescent signal was observed in all the control stigmas and in those located in the lower half of HD-stressed spikes. In contrast, significantly higher fluorescence was detected in HD-stressed papilla cells dissected from the upper florets of both varieties: a more than two- and ten-fold significant ($P \leq 0.05$) increase was observed in Plainsman V and Cappelle Desprez stigmas, respectively. The assessment of general cellular oxidative stress using H_2DCFDA indicated a good correlation with fertility loss, implying that the high level of oxidants detected in the stigmatic papillae was located in the top half of Cappelle Desprez spikes. The amount of oxidants in Plainsman V rose to a smaller extent, which did not lead to fertility loss.

The ROS metabolism in plant cells is an intricate network involving many enzymatic and metabolite elements (Noctor et al., 2015). The first type of ROS generated by the electron transport chain in mitochondria is superoxide (Rhoads et al., 2006). Unfortunately, the monitoring of superoxide with DHE and MitoSOX Red is impaired by the two-electron oxidation of these dyes by other oxidants (Wang et al., 2013), making these probes unsuitable for the selective detection of $O_2^{\bullet-}$. Nevertheless, the similar sensitivity and the organelle selectivity of these probes make them ideal for the quantitative comparison of the oxidants present in the mitochondria and cytoplasm (Zielonka and Kalyanaraman, 2010). The control level of these oxidants was 4-fold higher in the mitochondria compared to the cytoplasm, confirming the leading role of mitochondria in ROS generation. HD stress triggered a sharp rise in mitochondrial oxidant generation exclusively in Cappelle Desprez, while the cytoplasmic amount of these compounds rose significantly in both genotypes, irrespective of floret position. This suggests that although Plainsman V possesses a more efficient mitochondrial electron transport chain and/or ROS scavenging system than Cappelle Desprez, there are other cytoplasmic oxidant-generating mechanisms which produce similar levels of

oxidizing agents in the cytoplasm of both varieties. A very strong positive correlation was found between the cytoplasmic $O_2^{\bullet -}$ content and lipid peroxidation.

The amounts of highly reactive oxidative and nitrosative compounds, hydroxyl radical ($OH^{\bullet}$) and peroxynitrite ($ONOO^-$), respectively, were estimated using aminophenyl fluorescein (APF; Setsukinai et al., 2003). The results showed that the amount of these compounds elevated only in Cappelle Desprez papillae following HD stress, especially in those located in the top half of the spikes. In living cells, hydroxyl radical ($OH^{\bullet}$) is formed from H_2O_2 through the Fenton reaction catalyzed by iron and other transition metals (Rhoads et al., 2006). Despite its short lifetime, $OH^{\bullet}$ has a significant role in lipid peroxidation (Pham-Huy et al., 2008; Noctor et al., 2015). Peroxynitrite ($ONOO^-$), another highly reactive radical which can oxidize APF, is generated by the reaction of $O_2^{\bullet -}$ with NO (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). This short-lived reactive nitrogen species takes part in the oxidation and nitration of various molecules, such as DNA, lipids and proteins (Vandelle and Delledonne, 2011). The amount of hydrogen peroxide, a precursor molecule of $OH^{\bullet}$, closely mirrored the presence of the APF signal in the present experiment, which was not true for the precursors of peroxynitrite, NO and $O_2^{\bullet -}$. These data imply that the APF signal is potentially more indicative of hydroxyl radicals than peroxynitrite.

Superoxide is converted by superoxide dismutase enzyme (SOD) into hydrogen peroxide, a ROS with a long lifespan (Waszczak et al., 2018). H_2O_2 is able to diffuse through membranes either alone (Lim et al., 2016) or facilitated by aquaporins (Bienert and Chaumont, 2014), and may thus reach other parts of the cells. In the present study DHR 123 was used to monitor intracellular hydrogen peroxide, but as it also reacts with peroxynitrite (Crow, 1997), the results must be handled with care. DHR 123 diffuses freely into the cells, where it is oxidized to Rhodamine 123, which selectively stains mitochondria (Johnson et al., 1980). Hence, although the fluorescent signal is detected in the mitochondria, it represents the H_2O_2 and peroxynitrite concentration of the whole cell. The results showed that the cytoplasmic concentration of these compounds was only elevated in the top half of Cappelle Desprez spikes. In non-photosynthesizing cells, the hydrogen peroxide level may be elevated not only by mitochondrial activity, but also through the glyoxylate cycle in glyoxysomes. This pathway allows the turnover of membrane lipids as well as the synthesis of various hormones playing important roles in the stress response, like indole acetic acid, jasmonic acid and salicylic acid (Corpas et al., 2015). Higher expression of acyl-CoA oxidase, an enzyme that generates H_2O_2 in the glyoxylate cycle, was reported in drought-treated Arabidopsis plants (Bray, 2002). It can be hypothesized that the glyoxylate cycle became more active in the upper florets of Cappelle Desprez, contributing to the elevated H_2O_2 level in papilla cells. Increased amounts of apoplastic H_2O_2, determined with Ampliflu Red, were observed exclusively in treated Cappelle Desprez papilla cells. Apoplastic ROS is generated actively by enzymatic mechanisms, such as apoplastic polyamine oxidases and respiratory burst oxidase homologs localized in the plasma membrane (reviewed by Waszczak et al., 2018). According to the literature, apoplastic hydrogen peroxide is involved in drought and salt stress acclimation (Miller et al., 2010), although a more important role is proposed in intercellular signal transduction (Choudhury et al., 2017). The higher apoplastic levels of H_2O_2 in Cappelle Desprez may be explained by the occurrence of more severe water shortage in this genotype, which may induce elevated amounts of signaling molecules. Very strong correlations were found between the generation of intracellular H_2O_2, mitochondrial $O_2^{\bullet -}$, $OH^{\bullet}$, $ONOO^-$ and apoplastic H_2O_2.

The nitric oxide content and fertility of Cappelle Desprez were found to be lower in the top than in the basal halves of control spikes. After HD treatment, both values dropped in this

genotype, irrespective of floret position, while Plainsman V showed no significant change. An increasing number of scientific papers report the significance of NO in fertilization. Seligman and coworkers (2008) demonstrated the NO-generating activity of stigmatic tissue in Arabidopsis. Moreover, an Arabidopsis mutant proven to be defective in NO production showed reduced fertility, which was restorable using a NO donor compound (Guo et al., 2003). The present results confirm the proposed link between the HD stress-triggered drop in nitric oxide content and reduced fertility in monocotyledonous plants. This link may be the role that nitric oxide plays in pollen tube guidance. Prado and colleagues (2008) showed that NO acts as a negative chemotropic agent of pollen tube growth, providing a tool for maternal tissues to route pollen tubes towards the ovule. Positive correlation between fertility and NO content of stigmatic papilla cells indicates that this compound promotes successful fertilization in wheat.

HD co-stress induced peroxidation of membrane lipids

Oxidative damage during abiotic stress originates mainly from the alteration of lipids and proteins by oxidative compounds in the cell (Anjum et al., 2015). Lipid peroxidation was evaluated using a C11-BODIPY^{TM} probe, which showed a specific staining pattern localized in the membranes of papilla cells (Figure 8G). Treatment increased the oxidation of probes incorporated into the membrane in both genotypes, but to a different extent. On average, the proportion of oxidized C11-BODIPY^{TM} rose by 18% and 48% in Plainsman V and Cappelle Desprez, respectively (Figure 8H). Moreover, a very strong positive correlation was found between the cytoplasmic $O_2^{\bullet-}$ content and lipid peroxidation. It should be noted that the oxidation of Cll-BODIPY 581/591 can only be initiated by a variety of oxy-, peroxy-, or hydroxyl radicals, but not by superoxide, nitric oxide, or hydroperoxides (Pap et al., 2000), so the fluorescent signal intensity of the probe is not adequately proportional to lipid peroxidation. However, this fluorophore can be used to estimate the general oxidation of membrane lipids.

Correlation between reactive compounds and fertility loss

Very strong or strong negative correlations were found between the fertility ratio of the genotypes and the total ROS content (r = -0.85), extracellular H_2O_2 content (r = -0.94), intracellular H_2O_2 content (r = -0.84), $OH^{\bullet}$ and $ONOO^{-}$ content (r = -0.91), mitochondrial $O_2^{\bullet-}$ content (r = -0.91), cytoplasmic $O_2^{\bullet-}$ content (r = -0.47) and lipid peroxidation (r = -0.60). A very strong positive correlation was found between the cytoplasmic $O_2^{\bullet-}$ content and lipid peroxidation, while very strong correlations were detected between the generation of intracellular H_2O_2 (r = 0.98), mitochondrial $O_2^{\bullet-}$ (r = 0.93), $OH^{\bullet}$ $ONOO^{-}$ (r = 0.92) and extracellular H_2O_2. A moderate positive correlation (r = 0.55) was found between the fertility ratio and intracellular NO content of the papilla cells.

2.4. HD co-stress induced alterations in stigma proteome

Proteomic analysis (2D GE followed by LC-MS/MS and bioinformatics) of wheat stigmas revealed that Q5MCL9 Calreticulin-like protein appeared with HD co-stress in treated Plainsman V stigmas. In contrast, it was present both in control and HD co-stressed Cappelle Desprez stigmas on the same extent. This protein is transiently involved in the non-covalent folding, assembly and/or disassembly of other polypeptides or RNA molecules, including any transport and oligomerisation processes they may undergo, and the refolding and reassembly of protein and RNA molecules denatured by stress. The expression of M8ALQ3 Putative DNA repair protein RAD23 with a function of nucleotide-excision repair and proteasome-mediated

ubiquitin-dependent protein catabolic process changed in the same manner. The expression of M7ZLN1 Chaperonin CPN60-2, W5AA91 Importin subunit alpha and A5YVV3 Glyceraldehyde-3-phosphate dehydrogenase was upregulated by HD co-stress in treated Plainsman V, but their expressions were constant in the sensitive genotype. The first protein may prevent misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix, the second exports mRNA from nucleus in response to heat stress, the third protein catalyses the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes, including transcription activation, initiation of apoptosis and ER to Golgi vesicle shuttling. Interestingly W5FGH0 UTP--glucose-1-phosphate uridylyltransferase and Q9ZRB0 Tubulin beta-1/2/3/5-chain was downregulated by HD co-stress in the stigmas of Plainsman V, but in Cappelle Desprez it remained constant. However, a cluster of 6 protein spots lacking from HD co-stressed stigmas of the sensitive genotype were also sent for LC-MS/MS, no one of the proteins could be identified according to their peptide sequence.

2.5. HD co-stress induced alterations in stigma transcriptome

The number of HD co-stress induced, significantly overexpressed genes was nearly 10-fold higher in the sensitive Cappelle Desprez (1960) compared to the tolerant Plainsman V (190). Results of the GO term enrichment analysis showed that a large amount of genes involved in the following biological processes were upregulated in both genotypes: drought stress response, proline synthesis, redox processes, carbohydrate metabolism, iron ion homeostasis and lipid transport (Table 2; Figure 1-2, marked with red).

Table 2. Biological processes (GO terms) showing overrepresentation in upregulated genes following combined heat and drought stress treatment identified using a gene ontology enrichment analysis

Biological process	Significance (FDR corrected p value)	
	Cappelle Desprez	Plainsman V
Response to water	9.2058E-39	8.3251E-17
Proline biosynthetic process	3.0600E-9	4.3036E-5
Oxidation/reduction	7.4500E-14	9.0265E-5
Cellular carbohydrate metabolic process	1.0247E-6	1.2745E-7
Iron ion homeostasis	2.2709E-3	8.8756E-4
Lipid transport	4.3352E-13	3.6360E-3
Positive regulation of translation	2.2709E-3	ns
Signal transduction	3.1894E-6	ns
Spermine biosynthetic process	2.2678E-2	ns
Fatty acid beta-oxidation	3.8911E-7	ns
Asparagine biosynthetic process	1.8594E-4	ns
Nucleobase, nucleoside and nucleotide metabolic process	1.9024E-1	ns
Response to heat	ns	3.8452E-2
Cell redox homeostasis	ns	3.3066E-3
Protein folding	ns	3.0430E-11

Significance legend

p= 0,05 – 9,99E-5	
p= 1E-6 – 9,99E-10	
p= 1E-11 – 9,99E-20	
p= 1E-21 – 9,99E-30	
p= 1E-31 – 9,99E-40	

These functions can be considered as parts of the basic heat and drought stress response in the stigma tissue of winter wheat. Expression of the genes taking part in the regulation of gene expression, polyamine synthesis, signal transduction, nucleic acid metabolism, fatty acid beta oxidation and asparagine synthesis were induced exclusively in the stigmas of the sensitive genotype (Table 2; Figure 5, marked with green).

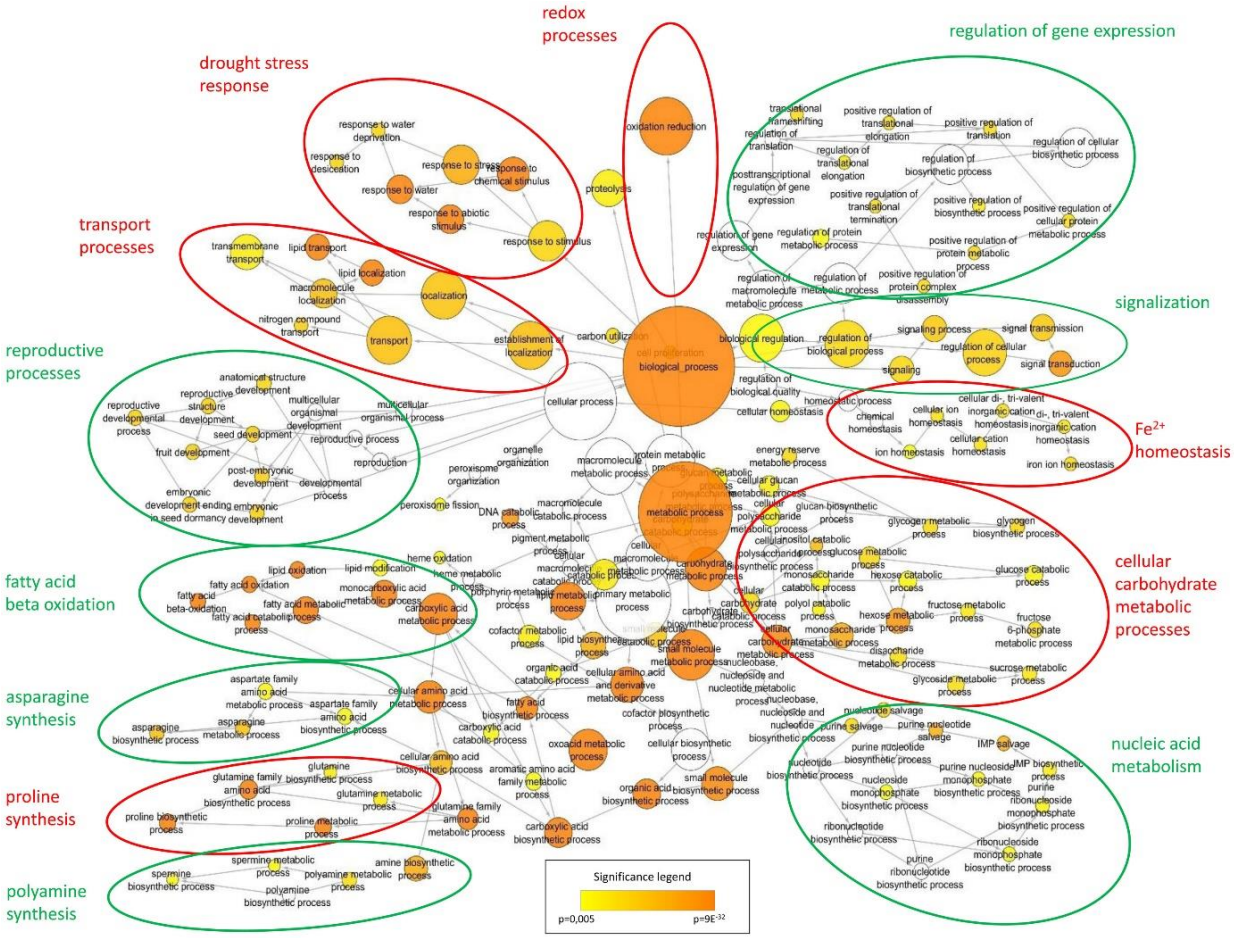

***Figure 5**. Genes expressed in HD co-stressed stigmas of the Cappelle Desprez genotype.*

The vast majority of these functions (excluding polyamine synthesis) are associated with general metabolism and cannot be linked with specific protective functions against heat or drought. In contrast, gene clusters with the functions of heat stress response, protein folding and cell redox homeostasis were enriched among upregulated genes only in the stigmatic tissue of the tolerant variety (Figure 6, marked with green). According to the results of the bioinformatics analysis, sensitive Cappelle Desprez activates a broad spectrum of genes with general metabolic functions in order to adjust metabolic processes to the changing environment, while tolerant Plainsman V upregulates less genes that are tightly linked with heat stress alleviation, antioxidant scavenging and structural repair of the damaged proteins. It can be assumed that the ability to avoid or repair the damage from heat and oxidative stress is a key factor for the enhanced drought and heat tolerance of Plainsman V.

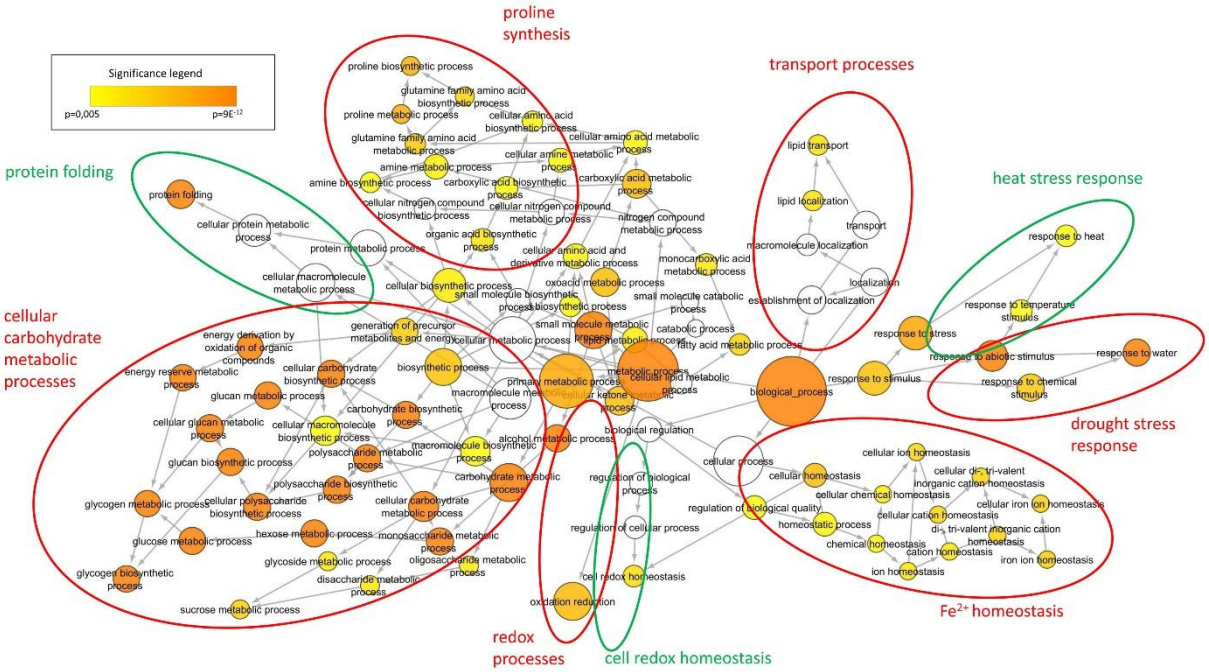

Figure 6. *Genes expressed in HD co-stressed stigmas of Plainsman V.*

A Real-Time PCR was performed to identify the expression level of a Wdhn13 gene coding for a dehydrin protein in control, drought-stressed and DH co-stressed flag leaves of Cappelle Desprez and Plainsman V genotypes. The expression of the Wdhn13 gene was induced both by water deprivation and HD co-stress exclusively in Plainsman V, however, compared to drought stress applied per se, the expression level was far greater in HD co-stressed flag leaves.

3. DISSEMINATION OF THE RESULTS

During this project we have published 1 research paper and the research paper
Fábián A., Sáfrán E., Szabó-Eitel G., Barnabás B., Jäger K.: *Stigma functionality and fertility are reduced by heat and drought co-stress in wheat* doi: 10.3389/fpls.2019.00244 has been accepted for publication in an open access journal Frontiers in Plant Science and going to be published in March 2019.

We were participating 4 national and 3 international congresses, presenting our results in 8 posters and 2 lectures.

Two manuscripts are in preparation that include data from this project:
Sáfrán E., Deák C., Fábián A., Kristóf Z., Barnabás B., Jäger K. Tolerance- dependent responses of photosystem II, antioxidant machinery and chloroplast morphology to heat and drought co-stress in wheat. Frontiers in Plant Science
Jäger K., Sáfrán E., Fábián A., Kristóf Z., Barnabás B., Deák C., Highlighting the factors critical for sustaining wheat production under heat and drought co-stress conditions. Plant Cell & Environment

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