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Research article

Effects of multiple abiotic stresses on lipids and sterols profile in barley leaves (*Hordeum vulgare* L.)

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ABSTRACT

Plants are usually exposed to several types of abiotic stress in regular field conditions. The lipid profile of barley homozygous lines exposed to drought, heat, salinity, and their combinations, was investigated in the present study. Free fatty acids, free sterols, and diacylglycerols were the most abundant classes (~8.0% of plant material). The genetic background significantly impacted the lipid composition rather than the treatments, and diacylglycerols were the only lipid class affected by salinity (1.84 mg/100 mg plant tissue; ~33% reduction). However, the genotype × treatment interaction analysis revealed that the lipid and sterol compositions depended on both genotype and environment. Our results suggest that inborn stress tolerance in barley is manifested by enhanced accumulation of most lipids, mainly sterols, especially in heat/drought-stressed plants. In addition, expression of the *LTP2* gene may be indirectly involved in the abiotic stress reaction of barley by mediating intracellular transport of some lipid classes.

1. Introduction

Several essential features of barley (*Hordeum vulgare* L.) contribute to its broad utilization in genetic studies. These traits include the crop's diploid nature with a high degree of inbreeding, its low chromosome number, and ease of cross-breeding and cultivation in a wide range of climatic conditions. Cultivated barley, ranked fourth in worldwide cereal production, is an extensively studied plant species in the field of genetics (Close et al., 2009; Fukuoka et al., 2010). Due to its geographic adaptability and natural tolerance to cold, drought, heat, or salinity, there is an increasing interest in identifying the stress response mechanisms in barley (Cominelli et al., 2013; Gürel et al., 2016). Numerous studies have focused on its response to abiotic stresses, e.g. drought (Pandey et al., 2015), salinity (Rejeb et al., 2014), heat (Mangelsen et al., 2011), and cold (Atkinson and Urwin, 2012). Most of these reports centered on the influence of a single stress; however, plants are usually exposed to multiple abiotic stresses in regular field conditions. For instance, drought is often accompanied by high temperature, and their combined effects have a greater impact on barley yield than the effects of a single stress alone (Prasad et al., 2011; Craufurd et al., 2013). Recent reports have revealed that the reaction of barley to combined stresses is rather unique and cannot be directly extrapolated from the response of the plant exposed to individual stress (Ahmed et al., 2013). However, it is known that salinity, drought, and high temperature can induce oxidative damage (Smirnoff, 1993; Cushman and Bohnert, 2000), and thus similar molecular responses may also occur in plants. In general, they led to a reduction of photosynthesis, transpiration, and other biochemical processes (Tiwari et al., 2010). Similarly, during exposition to high light, the excess of energy acting on the plant leads to disruption of cellular homeostasis, affecting i.a. lipid peroxidation and photoinhibition (Kalaji et al., 2012; Ksas et al., 2015). In fact, the available data on the combined effects of abiotic stresses on barley is limited, especially regarding changes in the leaf lipidome of barley under multiple abiotic stresses.

Improved high resolution and sensitivity in mass spectrometry (MS) have facilitated the identification and characterization of key compounds in biological processes, including metabolites, proteins, and lipids (Hill et al., 2014; Lee et al., 2012). Due to these recent technological advances, almost all modern lipidomic approaches utilize optimized and tailored MS-based methodologies (Horn and Chapman,

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2012) and these have been successfully applied to plant lipid research (Chalbi et al., 2015; Samarakoon et al., 2012).

Lipids are a group of biomolecules that are present in all plant tissues. They exert multiple roles and functions: constituents of the cell membrane, storage molecules of metabolic energy, and as signaling factors in response to stressors. Moreover, plant lipids are important sources of dietary phytochemicals for the food industry. Plants store lipids, usually as triacylglycerol (TAG) droplets. Apart from being a reservoir, TAG have other roles, such as host-pathogen interactions and abiotic stress responses (Xu and Shanklin, 2016). Considering the different lipid classes, sterols are of great importance as they exert a structural function in cell membranes, contributing to modulation of their fluidity. Sterols can exist in either in a free form or as esterified molecules with fatty acids or carbohydrates. Plant sterols are mainly composed of β-sitosterol, stigmasterol (24-ethylsterols), and campesterol (24-methylsterols). Apart from their structural function, sterols also play a regulatory role in plants. In fact, it seems that the balance among various types of sterols is a key element of numerous cellular responses (Schaller, 2003). Stress-induced changes impact the relative sterol composition, consequently modifying the properties of the cell membrane and its biological functions. Moreover, it is assumed that plant adaptation to stress may be determined by the ability of sterols to resist oxidation by reactive oxygen species (ROS) that are generated under various stress conditions (Piironen et al., 2000). ROS can react with unsaturated molecules, thus changing their structure and cellular functions (Anjum et al., 2015). In addition, campesterol has been reported to be the precursor for biosynthesis of brassinosteroids, a key group of steroidal hormones involved in plant growth and development (Vriet et al., 2015). All these facts justify the fundamental role that sterols play in the stress response.

On the other hand, different types of molecules in plant organisms can interact with lipids. For instance, LTP2 protein (lipid transfer protein 2, belonging to the family of pathogenesis-related (PRs) proteins) has the ability to bind not only linear lipid molecules (as in the case of LTP1), but also sterols. LTPs are involved in several biological processes, including *in vitro* lipid transference between membranes (Kader, 1996), deposition of cutin and wax (Sterk et al., 1991), and pathogen defense (Molina and Garcia-Olmedo, 1997; Stintzi et al., 1993). It is likely that they are also involved in plant growth and development (Kalla et al., 1994; Yeats and Rose, 2008).

We hypothesized that changes in the lipidome in barley green tissue are differentially expressed under conditions of combined stress compared to single stress. Secondly, we assumed that lipidome modifications are not only affected by stressor, but also are genetically determined and significance of environment/genetic impact was verified in this study. We also supposed that due to the role that the *LTP2* gene has in lipid transfer, it may have an essential role in modification of the lipidome in response to abiotic stress. Therefore, the objective of this study was to identify the multiple abiotic stress-induced modifications in different lipid classes in leaves of barley genotypes that differ in both the *LTP2* locus and stress adaptation. Detailed evaluation of phytosterols was done and the effects of the changes observed in stressed plants were discussed.

2. Materials and methods

2.1. Reagent and Solvents

All chemicals and solvents were of analytical grade. Water (≥99.8%) and HPLC-grade methanol (≥99.9%) were acquired from Merck (Darmstadt, Germany), while bidistilled water (100%) was supplied by Panreac (Castellar del Vallès, Spain). The standard mixtures of free fatty acids (GLC 406) were purchased from Nu-Chek (Elysian, MN, USA). Triolein (≥99.0%, CAS 122-32-7), tripalmitin (≥99.0%, CAS 555-44-2), tristearin (≥99.0%, CAS 555-43-1), 1,3-diolein (≥99.0%, CAS 2465-32-9), 1,2(3)-dipalmitin (≥99.0%, CAS 26657-95-4), cholesteryl palmitate (≥97%, CAS 601-34-3), 1-oleoyl-*rac*-glycerol (≥99.0%, CAS 111-03-5), methyl tridecanoate (≥99.5%, CAS 1731-88-0), 5α-cholestan-3β-ol (dihydrocholesterol, ≥95%, CAS 80-97-7), 5-cholesten-3β-ol (cholesterol, ≥99%, CAS 57-88-5), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol, ≥98% CAS 83-48-7), 3β-stigmast-5-en-3-ol (β-sitosterol, ≥95%, CAS 83-46-5), 24α-methyl-5-cholesten-3β-ol (campesterol, ≥65%, 474-62-4), lup-20(29)-ene-3β,28-diol (betulin, ≥98%, CAS 473-98-3), and 5α-cholestane (≥97%, CAS 481-21-0), were purchased from Sigma (St. Louis, MO, USA). The purity of sterols was controlled by gas chromatography-flame ionization detector (GC-FID). Grade 1 filters (70 mm diameter) were used (Whatmann, Maidstone, England). Stanol standards were obtained by hydrogenation of sterols, as reported in our previous work (Inchingolo et al., 2014). Hexamethyldisilazane, trimethylchlorosilane, and palladium (purity: 99.99%) were bought from Sigma, while dried pyridine was supplied by Carlo Erba (Milan, Italy). The silylation mixture was prepared with dried pyridine, hexamethyldisilazane, and trimethylchlorosilane, at a ratio of 5:2:1 by volume.

2.2. Plant materials

The plant material consisted of two different types of homozygous spring barley lines and their parental forms – eight individuals in total. The development and characteristics of the lines were described by Mikołajczak et al. (2016a, 2016b). Briefly, parental genotypes with contrasting growth habit (MPS37-erect and MPS106-prostrate) of backcross (BC) lines were derived by single seed descent technique from the hybrids between variety Pomo and Maresi, which were chosen in favor of a large phenotypic diversity. Maresi is a two-rowed, hulled, semi-dwarf (*sdw1/denso*), brewing cultivar, whereas Pomo is a six-rowed, hulled, fodder cultivar. Hybrids MPS37 (donor) × MPS106 (recurrent) were backcrossed with the MPS106 line up to BC_6 generation supported by SNP markers targeting in the *LTP2* allele variation. On this basis, unique BC lines MPW14/19 and MPW15/14 were included in the present study. MPW15/14 carries the same allelic form of the *LTP2* gene as the initial parent MPS106, but is the most similar line to MPS37 from a genetic standpoint. In turn, MPW14/19 has the same *LTP2* gene allelic form as MPS37 (Mikołajczak et al., 2016b).

Recombinant inbred lines (RILs F_{10}/MCam) were also used in the present study. They were obtained from cv. Maresi and Cam/B1/CI08887/CI05761 (CamB1) cross combination. CamB1 is a Syrian inbred line adapted to the water deficiency conditions (Górny, 2001). Stability analysis (Mikołajczak et al., 2016a), together with genotypic and phenotypic data, allow for line selection with both advantageous parental genotypes alleles: the earliness from the Syrian line and the semi-dwarfness from cv. Maresi with simultaneous stable grain yield under water deficit conditions. On this basis, MCam53 (RIL stable with respect to yield) and MCam71 (extensive RIL, better grain yield in stress conditions compared to the control) were chosen for the study.

2.3. Methods

2.3.1. Abiotic stresses application

All experiments were conducted in growth chambers under fully controlled conditions. Seeds were sown in pots filled with a mixture of peat and sand (3:1, w/w) and plants were cultivated in optimal conditions: soil moisture 70% FWC (filed water capacity), 22 °C/18 °C day/night, air humidity of 50–60%, and a photoperiod of 16/8 h of light/dark. All abiotic stresses were imposed in the tillering stage (21 BBCH scale) and maintained for 14 days: (i) water scarcity was established at 20% FWC (Samarah, 2005) and soil moisture in each pot was controlled gravimetrically by weighing and, additionally, volumetrically (if necessary) using the FOM/mts device (Mikołajczak et al., 2017; Ogrodowicz et al., 2017); (ii) salinity stress: pots were watered with a NaCl solution to obtain a final NaCl concentration of 250 mM/dm^3 (Fayez and Bazaid, 2014) measured by FOM/mts; (iii) temperature

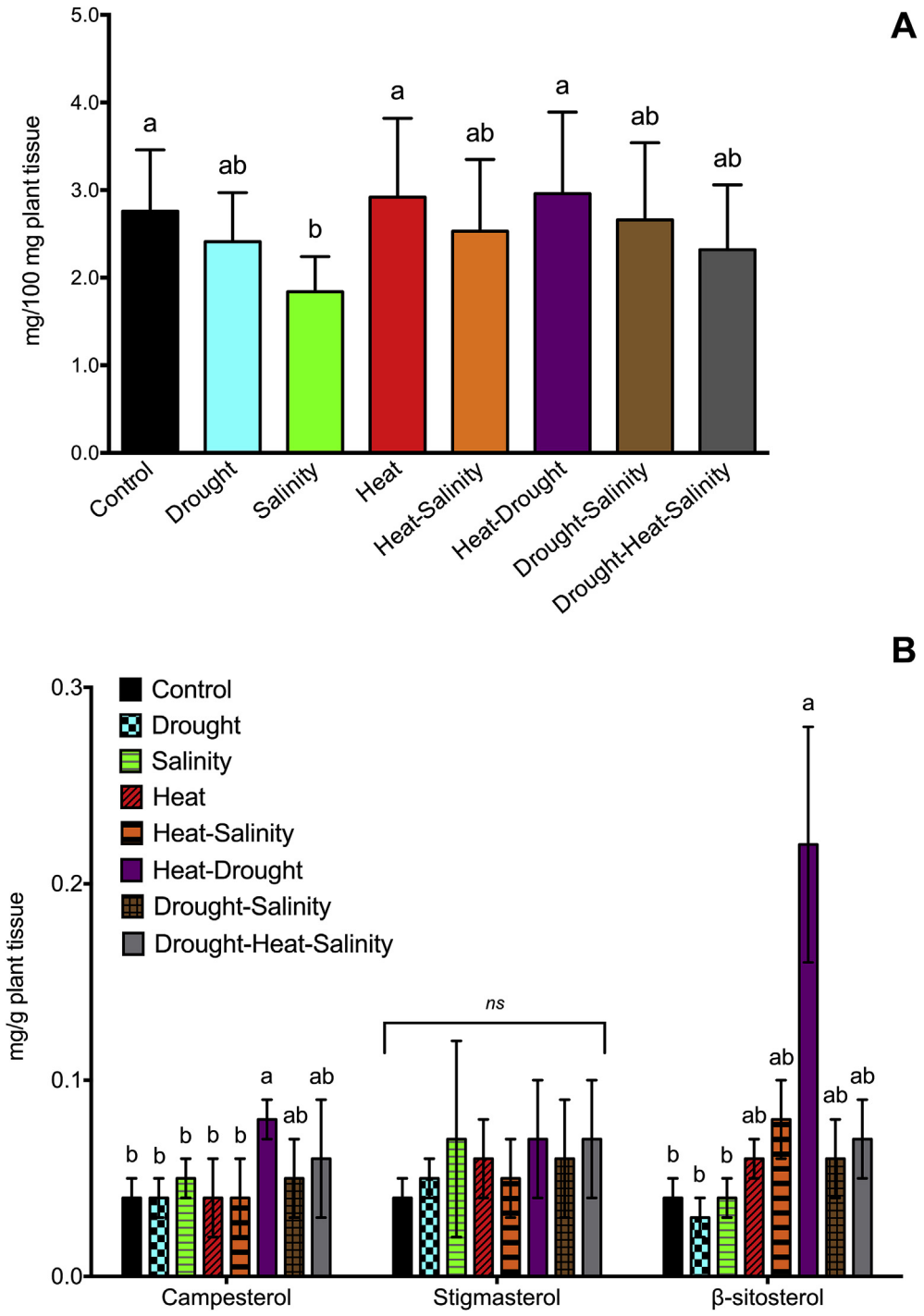

Fig. 1. Effects of abiotic stress conditions on diacylglycerols (DAG; mg/100 mg plant tissue) (**A**) and sterols (mg/g plant tissue) (**B**). Results are expressed as mean and standard deviation (SD). Different letters (a–b) denote statistically different means (Tukey's test; $P \leq 0.05$); ns, not significant. When bars report different letter, significance differences were detected. The abiotic effect was analyzed for each sterol.

stress was 30 °C during the day and 10 °C at night (Kalaji et al., 2011). In total, eight different treatments were performed: control (C), drought (D), salinity (S), heat (H), heat-drought (HD), heat-salinity (HS), drought-salinity (DS), and drought-heat-salinity (DHS). In the last day of stresses application, barley leaves (in duplicate) were collected for lipids and sterols analysis, pulverized in liquid nitrogen, and frozen at −70 °C in a 1.5 mL tube until extraction. Each replication consisted of leaves collected from three plants from one pot.

2.3.2. Lipid extraction

The lipid extraction was carried out as suggested by Folch et al. (1957). In brief, about 200 mg of barley leaves were added with an internal standard (50 μg of 5α-cholestane) and 7.5 mL of chloroform:methanol (1:2, v/v). The samples were vortexed for 2 min and, after sonication (10 min), 2.5 mL of chloroform and 4.5 mL of KCl (1 M) were added. The samples were centrifuged (1800 × *g*) and the organic phase was withdrawn and evaporated under vacuum. The extracted lipid matter was diluted in chloroform and injected into a GC-FID.

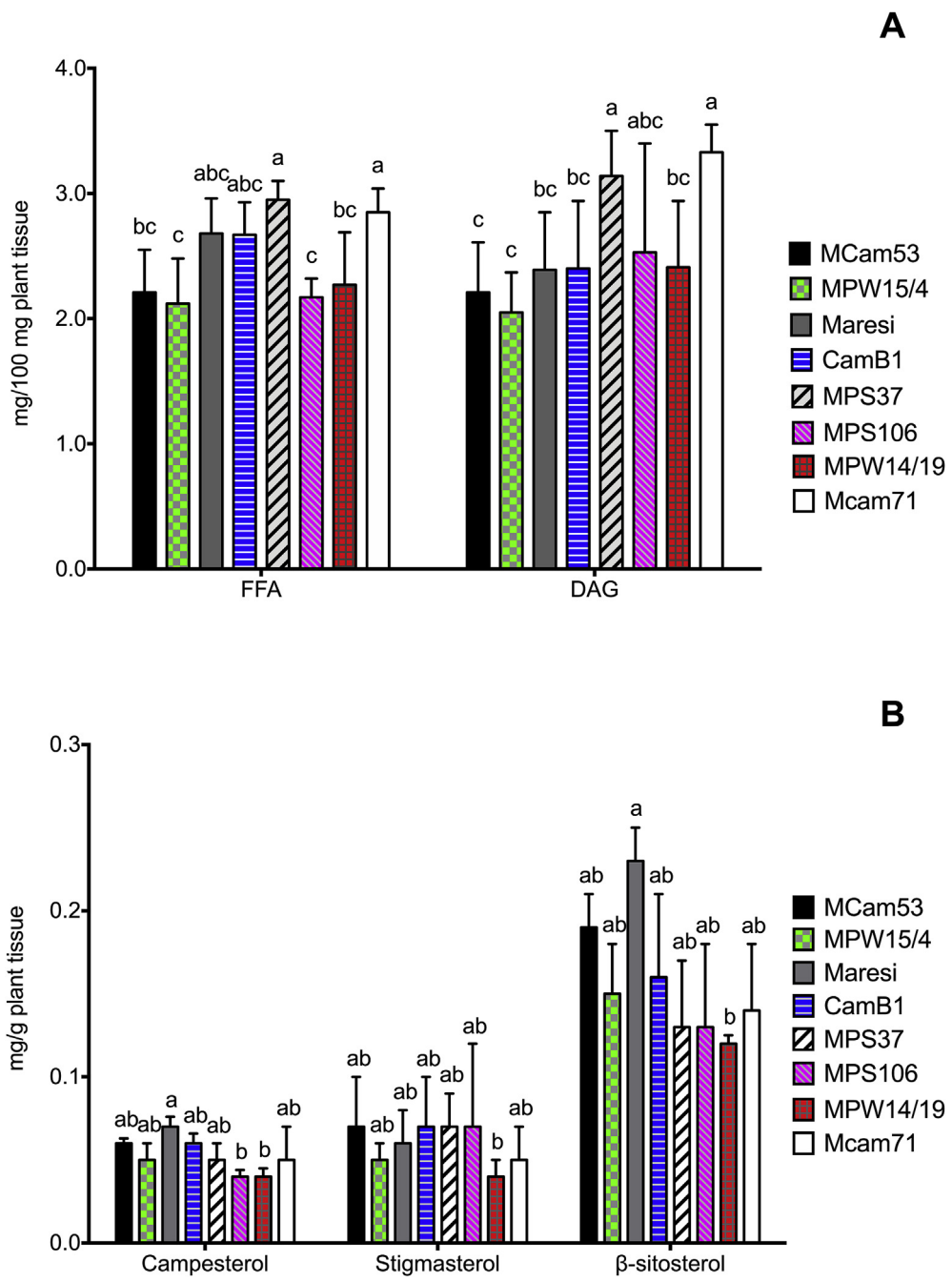

Fig. 2. Effects of genotypes on free fatty acids (FFA; mg/100 mg plant tissue) and diacylglycerols (DAG; mg/100 mg plant tissue) (**A**) and sterols (mg/g plant tissue) (**B**). Results are expressed as mean and standard deviation (SD). Different letters (a–c) denote statistically different means (Tukey's test; $P \leq 0.05$). The significant differences were separately analyzed for FFA, DAG and each single sterol.

2.3.3. Lipid profile

The lipid profile of the extracted fat was obtained as reported by Gallina Toschi et al. (2014). One μL of lipid extract dissolved in chloroform (2%, w/v) was injected into a GC-FID (GC Shimadzu QP 2010 Plus; Kyoto, Japan). A fused silica capillary column (Restek RTX-5, 10 m × 0.25 mm i.d. × 0.1 μm film thickness; Bellefonte, PA), coated with 95% dimethyl- and 5% diphenylpolysiloxane, was used. The temperature was programmed from 100 °C to 350 °C at 6 °C/min. The injector and FID temperatures were set at 350 °C. The injection was performed in the split mode (1:50), and helium was used as carrier gas at a flow of 1 mL/min. The different lipid classes (free fatty acids (FFA), free and esterified sterols (STE, E-STE), monoacylglycerols (MAG), diacylglycerols (DAG), and triacylglycerols (TAG) were identified by comparing their retention time with those of the corresponding chemical standards (see section Reagent and Solvents) and by injection in gas chromatography coupled with mass spectrometry (GC/MS) for more accurate recognition. Data are reported as mean (mg/100 mg plant tissue) of at least three independent replicates (n = 3).

2.3.4. Determination of total sterol composition

The sterol matter was first isolated (Menéndez-Carreño et al., 2012) and then analyzed by Fast-GC/MS (GC Shimadzu QP 2010 Plus; Kyoto, Japan) (Cardenia et al., 2017) (Fig. S1, S2, S3A-F). Briefly, about 200 mg of leaves were added with the internal standard (betulin; 50 μg) and 3 mL of a KOH solution in methanol (4 mol/L) containing BHT (5 mg/mL) to carry out cold saponification lasting 18 h at room

temperature under darkness. Thereafter, 10 mL of dichloromethane and 5 mL of citric acid (0.1% double distilled water) were added to each tube and mixed thoroughly using a vortex. Samples were then centrifuged at 2500 × *g* for 10 min; after phase separation, the organic phase was isolated and washed with 5 mL citric acid (0.1%, v/v) solution. The dichloromethane phase was evaporated at 40 °C with nitrogen and the residues were dissolved in 5 mL of dichloromethane. The extracted unsaponifiable matter was silylated and used for determination of sterol composition as reported by Inchingolo et al. (2014). The acquisition and integration modes were Full Scan (TIC) and Single Ion Monitoring (SIM), respectively. Sterols were recognized and quantified by their corresponding characteristic ions: 343 *m/z* (campesterol), 83 *m/z* (stigmasterol), 396 *m/z* (β-sitosterol), 488 *m/z* (sitostanol), and 459 *m/z* (campestanol). The identification of sterols was carried out by comparing mass spectra and retention times with those of the corresponding chemical standards. Data are reported as mean (mg/g plant tissue) of at least three independent replicates (n = 3).

2.3.5. Statistical analyses

Data are reported as mean ± SD (three independent replicates). One-way ANOVA, followed by Tukey's honest significance test, was carried out at a 95% confidence level ($p \leq 0.05$) to separate means of parameters and interactions that were statistically different. Factorial analysis of variance (ANOVA) was carried out to investigate the impact of various treatments on the main lipid classes and sterols, as well as their interactions. Principal component analysis (PCA) was also performed on all data sets to better understand the variability of the data. The hierarchical cluster analysis by the method of further neighbor, using the squared Euclidean distance as the measure of dissimilarity, was done. Statistical analysis of the data was carried out using IBM SPSS Statistics v.25 (2018, IBM-SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

The main lipid classes were well separated by GC-FID analysis. In general, FFA, STE and DAG were the most abundant, while TAG and MAG were less than 0.03% (w/w) of plant material.

The abiotic stresses did not significantly affect the composition of lipid classes except for DAG, which significantly ($p < 0.05$) decreased when salinity was applied (Fig. 1A). On the other hand, the highest amounts of DAG were found in control, heat, and heat-drought conditions, while the other five treatments were associated with similar, lower values. The sterol matter was mainly composed of β-sitosterol (0.13–0.26 mg/g plant), campesterol (0.04–0.08 mg/g plant tissue), stigmasterol (0.04–0.07 mg/g plant), sitostanol (0.01–0.03 mg/g plant), Δ5-avenasterol (< 0.03 mg/g plant), and campestanol (< 0.01 mg/g plant). Among treatments, the greatest increase of sterols in leaves was observed under heat-drought stress; in particular, higher amounts of campesterol and β-sitosterol were accumulated (Fig. 1B). On the contrary, the content of stigmasterol and Δ5-avenasterol was not significantly affected by abiotic stress. In general, a low sterol level was found in samples collected from control conditions.

The main effect of genotypes was also analyzed. The genetic background had a significant impact on lipid composition (Fig. 2A). FFA were higher in MPS37 (2.95 g/100 g plant) and MCam71 (2.83 g/100 g plant) than in MPW15/4 (2.12 g/100 g plant) and MPS106 (2.17 g/100 g plant). DAG displayed a similar behavior, since MCam71 with MPS37 showed the highest content (3.33 and 3.14 g/100 g plant, respectively), while MPW15/4 had the lowest level of DAG (2.05 g/100 g plant). Moreover, the MCam71 line exhibited the lowest accumulation of MAG (0.21 g/100 g plant), whereas the other genotypes reported higher amounts. The TAG content did not vary across the barley genotypes analyzed. Although non-significant variations in total quantity of STE and E-STE were observed, significant differences were found in sterol composition (Table S1). Maresi had the highest sterol level, in particular campesterol (0.07 mg/g plant) and β-sitosterol (0.23 mg/g plant). MPW14/19 showed the lowest amount of sterols, while CamB1 and MPS106 displayed the highest accumulation of stigmasterol (Fig. 2B).

To better understand these results, interactions between genotypes and each abiotic stress condition were also investigated (Table S2). In general, statistical analysis revealed a significant interaction of main factors for campestanol (< 0.001), stigmasterol (< 0.001), MAG (0.05), STE (0.01), DAG (0.001), E-STE (0.01), and TAG (0.02). Overall, in control treatment, FFA were higher in MCam71, while MPW15/4 had the lowest amount of FFA; DAG showed a similar trend in these two genotypes. Regarding sterol composition, only campestanol was significantly higher in Maresi. Drought did not affect the sterol composition, but the content of FFA and DAG was greater in MPS37 and MCam71, respectively. For salinity, no significant interactions were found. Heat enhanced accumulation of MAG and Δ5-avenasterol in CamB1, as well as sterols (in both free and esterified forms) in MPW15/4; on the other hand, TAG were higher in Maresi after exposure to heat treatment. Combined heat-salinity led to a significant ($p < 0.05$) increase of MAG in CamB1 and E-STE in Maresi. Combined heat-drought influenced the lipid composition of barley leaves; in fact, CamB1 had a higher amount of stigmasterol, MAG, and TAG, while DAG and campestanol were greater in MPS106 and Maresi, respectively. Drought-salinity mainly increased the content of FFA, total sterols (STE and E-STE), DAG, and TAG in MPS37. Finally, drought-heat-salinity led to the increase of campesterol, stigmasterol, β-sitosterol, MAG, DAG, and TAG in MCam53 (Table S2).

To better clarify the relevance of interactions between genotypes and abiotic stress conditions on the lipid composition, and to better classify the behavior of different genotypes when subjected to diverse abiotic stresses, principal component analysis (PCA) of all data was performed. The first three principal components reached 70.79% of variance, but considering that only Δ5-avenasterol was correlated to Component 3, it was decided to reduce it to two principal components. Component 1 explained 37.67% of variance, being more correlated to the sterol composition (mainly campesterol, campestanol, stigmasterol, sitostanol, and β-sitosterol) except for Δ5-avenasterol. Component 2, which explains 22.51% of variance, was related to the main lipid classes, in particular FFA, DAG, STE, and E-STE (Fig. 3).

Considering the Biplot (Fig. 4), all experimental groups were separated into different areas. Since all samples were studied as crossed interaction between the genotype and the abiotic stress conditions, they are indicated in the Biplot as genotype-abiotic stress abbreviations. Under C treatment, MPW14/19-C, MPS37-C, MCam71-C, and MCam53-C were inversely correlated with sterol composition, while MPW15/4-C and CamB1-C were inversely correlated with lipid classes. MCam53-HD was mainly characterized by Δ5-avenasterol, whose vector separated MCam71-S, MPW14/19-HD, MPS106-DHS, and MCam53-HD. Moreover, Maresi-DS, MCam53-HS and MPS106-S were more correlated with Δ5-avenasterol than to the other sterols. Maresi-HD was characterized by β-sitosterol and campesterol; while Maresi-C by MAG. TAG, DAG, and FFA defined Maresi-HS, MPS106-HD and MPS37-D, respectively.

Finally, hierarchical cluster analysis was carried out (Fig. 5). Two main clusters were created where many different sub-clusters were designed. In particular, MCam53 and MCam71 were significantly influenced by DHS condition, while the other treatments were located in different clusters. MPW15/4 was affected only by H abiotic conditions. The lipidome of Maresi was mainly impacted by DS, S, and D treatments. On the other hand, no effects due to abiotic conditions were reported in CamB1 for HD, HS, D and H, being all grouped in the same cluster together with the C group. MPS106 was separatedwith D, H, S, and their combined effects, except for HD and DS. Regarding MPW14/19, D, HS, and S were in the same cluster and detached from other cluster groups. In the case of MPS37, its interaction with S was completely isolated from the other abiotic conditions, among which DS was,

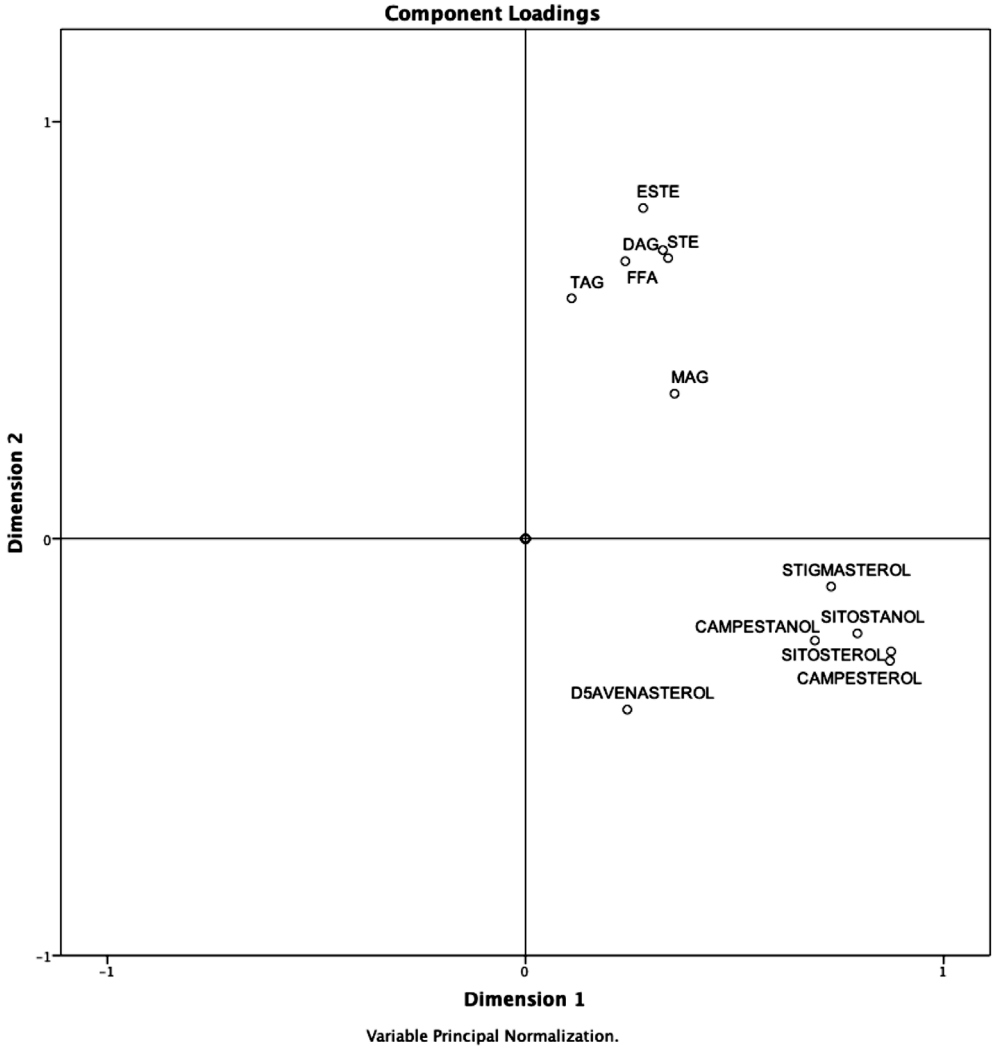

Fig. 3. PCA loading plot of main lipid classes and individual sterols.

in turn, separated from the HS, H, and HD groups.

4. Discussion

Drought, heat, and salinity are among the main abiotic stresses that limit the growth and productivity of crop species (Barnabás et al., 2008). Among cereals, barley is considered as an ideal model plant for deciphering the abiotic stress response due to its short growth period, early maturing, diploid and self-pollinating characteristics, as well as its increased adaptation to poor environmental conditions (Munns and Tester, 2008; Honsdorf et al., 2014). Our previous studies on homozygous lines obtained from Maresi and Pomo cross combination showed that the differences at the proteomic and morphological levels are most visible in the tillering stage (Kuczynska and Wyka, 2011; Kuczyńska et al., 2012). In other studies, we found that the water deficit was much more constrained to RILs populations in the late stage compared to the earlier development stages (Mikołajczak et al., 2017; Ogrodowicz et al., 2017). For this reason, in the present investigation, experiments involving the application of various stress conditions were planned to include plant tillering. In this study, we focused on evaluation of changes in the main lipid classes (TAG, DAG, and FFA). We found that combined heat and drought led to the highest level of TAG in CamB1 compared to other genotypes. According to our previous quantitative trait loci (QTL) mapping, conducted on MCam population (consisted of 100 RILs) under different water regimes, CamB1 Syrian parental genotype contributed to F_{10} progeny numerous alleles increasing yield components under drought (Mikołajczak et al., 2017, 2016a). Comparing those findings with the present results, we can affirm that the positive genetic impact of the aforementioned CamB1 alleles in response to abiotic stress may be partially explained by higher TAG accumulation in leaves. This possibility is based on literature data indicating that total leaf TAG content appears to be closely related to plant stress tolerance. Yang and Benning (2018) reported that reduced TAG biosynthesis results in hypersensitivity toward abiotic stresses, since TAG in cytosolic lipid droplets is involved in membrane integrity, fluidity, and growth under abiotic stress conditions (e.g. source of oxylipins from polyunsaturated fatty acids).

Herein, salinity alone affected the lipid composition in terms of DAG, which significantly decreased. As reported in the literature, DAG may originate from TAG through lipase-dependent activity (Cardenia et al., 2018). In fact, TAG contains three fatty acids (FA) esterified to glycerol and lipase hydrolyzes FA, thus generating DAG, MAG, FFA, and glycerol. In addition, DAG can derive from phospholipids, phosphatidic acid, and MAG through the activity of phospholipase/transferase, phosphatase, and acyltransferase enzymes, respectively (Eichmann and Lass, 2015). DAG represent a major structural component of plant membranes involved in their dynamics (such as membrane fusion in cell division) and thus a reduction in DAG may impact plant growth. A decrease in formation of DAG could be ascribed to inhibition of the growth of shoots, which is partially due to a loss of cell wall

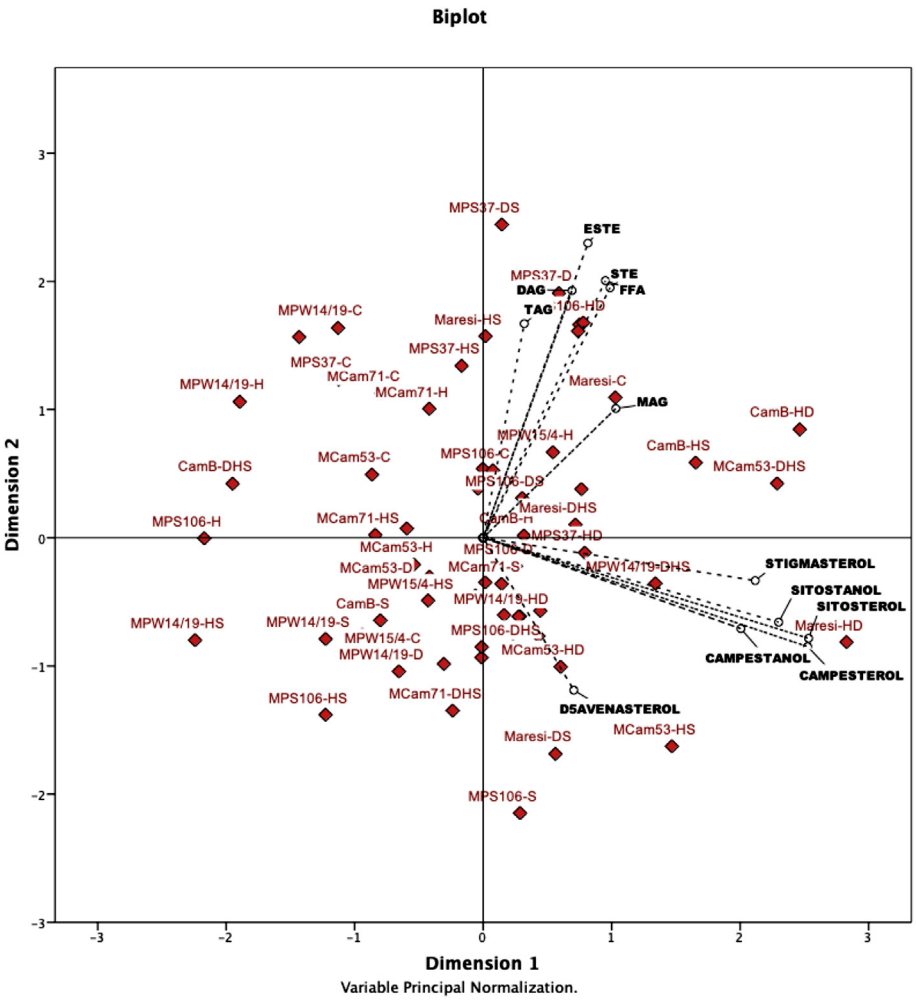

Fig. 4. PCA score plot of main lipid classes, individual sterols, and combined treatments.

extensibility and in general to changes in cell wall structure (Le Gall et al., 2015). It should be pointed out that the DAG content of plant cells is usually low, since it is rapidly converted to phosphatidic acid (PA), but during conditions of particular environmental stimuli its presence is necessary (Dong et al., 2012). Some reports suggest that the decrease in DAG can be assigned to the downregulation of the phosphatidylcholine (PC)/phospholipase (PLC) pathway; a significant decrease of DAG without an increase in PA was reported in *Arabidopsis* treated with $AlCl_3$ (Pejchar and Martinec, 2015). Additionally, environmental stress can induce the transcription of a number of genes encoding enzymes involved in catalysis of PA to DAG, reducing the conversion of diacylglycerol pyrophosphate (DGPP) to PA and consequently of PA to DAG (Dong et al., 2012). In agreement with the literature, our results demonstrated that the content of DAG changes in response to different environmental conditions, even though the mechanism involved remains unclear.

On the other hand, combined drought and salinity led to the highest amount of FFA in MPS37. This barley line was previously chosen for development of backcross population as a donor of good parameters associated with yield across experiments under field and greenhouse conditions (Kuczyńska et al., 2014). It is well known that biotic and abiotic stresses in plants induce oxidative cascades involving FFA, promoting the synthesis of stress-related hormones. Overall, FFA can give rise to important intracellular signals, so that plants with increased levels of FFA can better cope with adverse environmental conditions. This fact could render MPS37 a potentially better adapted genotype to multiple stresses. In contrast, MPW15/4, having a genetic background that is most similar to MPS37 but with a different allelic form of the *LTP2* gene (Mikołajczak et al., 2016b), was characterized by the lowest levels of FFA. Interestingly, the second lowest FFA level was observed in MPS106, which has the same allelic form of the *LTP2* gene as MPW15/4. These results suggest that, since the *LTP2* gene mediates FFA transference, and thereby effective signal transduction in cells, it can probably indirectly influence the molecular response of barley to abiotic stress, even though this aspect requires further investigation.

It is well known that plant sterols are functional components of the membrane lipid bilayer, since they regulate its fluidity and properties as well as metabolic processes such as signal transduction events for cell division, membrane permeability, and activity of membrane-bound enzymes (Kumar et al., 2015). In plants, sterols are present in their free (STE) or conjugated forms (such as steryl esters, E-STE); conjugated sterols are ubiquitously found in plants, but their relative contents vary widely among species and their profile may change in response to both developmental and environmental cues (Ferrer et al., 2017). Recent evidences support the involvement of conjugated sterols in the response of plants to stress, but few studies have been published about their metabolism; in fact, only a small number of genes encoding enzymes implicated in conjugated sterol metabolism have been cloned (Ferrer et al., 2017). It should be pointed out that, in our study, the STE and E-STE classes did not display significant variations; however, when the composition of total sterols was investigated, significant differences were found. It might be possible that the presence of some interferences affected the STE and E-STE classes, increasing the dispersion of data and thus leading to non-significant differences. This problem was

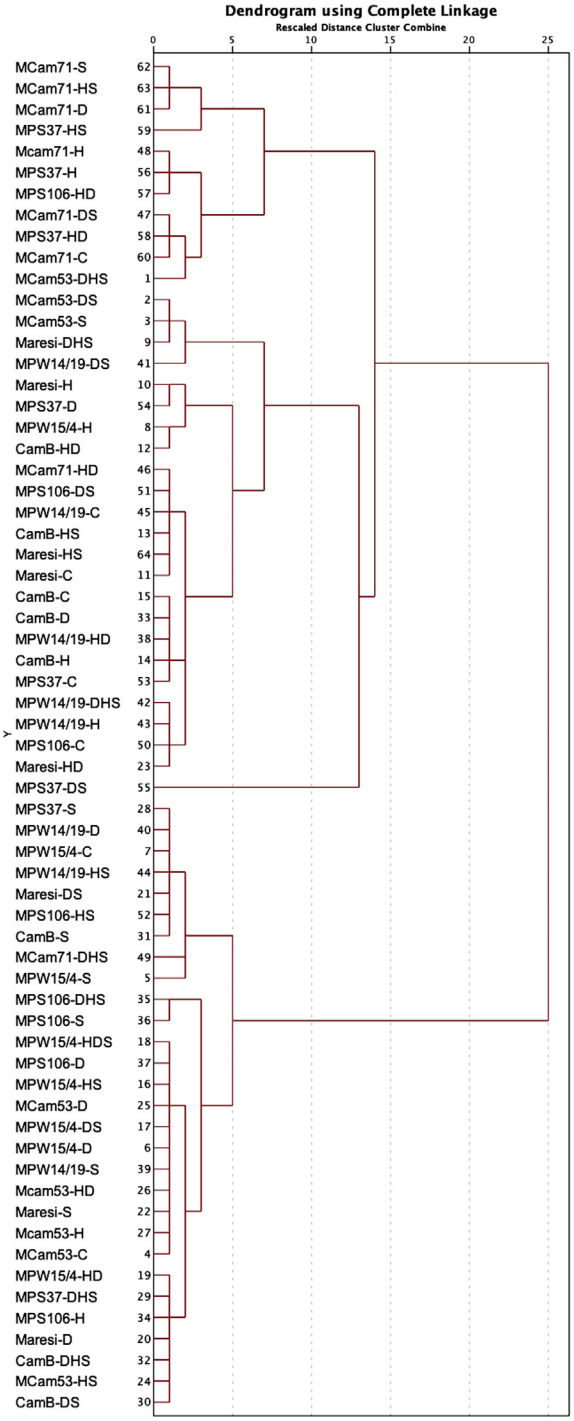

Fig. 5. Dendrogram resulting from cluster analysis of lipidomic evaluation.

overcome when GC/MS was used to reveal the exact composition of the sterol fraction. In agreement with the literature, the predominant sterol was β-sitosterol. As reported by Wang et al. (2017), cycloartenol acts as a sterol precursor from which ethyl-sterols (such avenasterol, fucosterol, β-sitosterol, and stigmasterol) and methyl-sterols (such as campesterol) are synthesized in plants. Among the barley genotypes studied, MCam53 was characterized by the highest level of most lipids and sterols. Moreover, MCam53 was found to exhibit the greatest increase in β-sitosterol and campesterol in response to heavy stress (DHS). Of note, in our previous study, MCam53 was stable considering grain yield in field conditions, even when rainfall was limited (Mikołajczak et al., 2016a). It is believed that the balance of β-sitosterol and campesterol strongly modulates cell growth and membrane integrity in higher plants, also in constrained environments (Schaller, 2003). Interestingly, Kumar et al. (2015) noticed the enhanced accumulation of β-sitosterol in a drought tolerant rice cultivar compared to a drought sensitive cultivar. In addition, our results showed that the lipidome of MPW14/19, characterized by the lowest level of campesterol, stigmasterol, and β-sitosterol, was highly susceptible to all stress treatments, being grouped in different clusters from control as revealed by hierarchical clustering. Combining all these facts, it is tempting to speculate that the enhanced level of both β-sitosterol and campesterol associated with stress may determine the resistance of barley to drought and other abiotic stresses. This possibility is justified since increased sterol content improves membrane rigidity, which is one of the main plant responses in conditions of abiotic stress; an increase in membrane rigidity may also reflect a reduction of membrane permeability and transpiration (Dufourc, 2008). Wang et al. (2017) found out that increased sterol matter was positively correlated with salinity tolerance and heat acclimation in hard fescue. The characteristics of the MCam53 genotype in terms of lipid and sterol profiles may explain its inborn grain stability observed in our previous experiments. Convincingly, this RIL may be an excellent germplasm resource for improvement of barley to unfavorable abiotic conditions. On the other hand, MCam71, previously characterized to be extensive in water deficiency (Mikołajczak et al., 2016a), showed a lower content of some sterols compared to MCam53, while hierarchical clustering revealed no response in the lipidome across all treatments. In this case, probably an alternative mechanism provokes the better yielding in stress conditions. Interestingly, PCA revealed that Δ5-avenasterol acts in a different way compared to other sterols, which was mainly correlated with MCam53 (under HD treatment). Since stigmasterol, β-sitosterol, and campesterol are reported to be, in general, involved in a plant's reaction to stress, the stress-induced changes in Δ5-avenasterol may suggest that it has a unique role in the response of barley to abiotic stresses, which has not been recognized to date.

The present study confirms that abiotic stress conditions, such as combined heat and drought (HD), significantly increase the sterol content. A similar behavior was reported by Kumar et al. (2015) in rice seedlings subjected to drought stress. Both drought and high temperature stimulate the generation of reactive oxygen species in plant cells, which inhibit metabolic enzyme activities, increase lipid oxidation, and damage nucleic acids with a consequent reduction of the relative water content in leaves (Hussain et al., 2018). Drought causes destruction of cell membranes, and thus sterols act to preserve the integrity of cell membranes (Kumar et al., 2015). On the other hand, when the severity of drought raises, Kumar et al. (2018) found an increase in the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and phosphoserine aminotransferase (PSAT), which are involved in sterol biosynthesis and turnover, respectively. Furthermore, sterols are crucial regulators of membranes, thereby contributing to rigidity of the plasma membrane (Mamode Cassim et al., 2019). In addition, *At-cyp710A1* gene appears to play a key role in membrane permeability and fluidity, by catalyzing the conversion of β-sitosterol into stigmasterol (Senthil-kumar et al., 2013). Indeed, the stigmasterol/β-sitosterol ratio in the plasma membrane may affect the reaction of cells to various stress factors. Moreover, a high level of stigmasterol can modify the fluidity and permeability of membranes, resulting in restricted release of substances into the apoplast (Senthil-kumar et al., 2013). Based on our results, CamB1 showed the highest increase in the level of stigmasterol during HD treatment. It must be noted that CamB1 originates from a warm and arid region, hence this phenomenon is justified and provides a new explanation about the background of its stress tolerance. Our findings show that the sterol content increased in some homozygous barley lines under abiotic stress, which may result in enhanced membrane rigidity in plants (Zheng et al., 2011). In fact, drought and heat affect photosynthesis, as well as the absorption of water and solutes; to avoid dehydration, plants respond by accumulation of sterols in order to harden the cell wall, which leads to concomitant reduction

of membrane permeability and transpiration (Hussain et al., 2018).

In conclusion, this study demonstrates discrepancies in modulation of the lipidome in barley leaves via combined stresses, particularly for drought and heat, compared to a single stressor. The results confirm that β-sitosterol is a major component of the sterol fraction of barley leaves. Considering different genotypes and treatment conditions, it would appear that variations in sterol composition are dependent on both the genotype and environmental factors, while changes in the main lipid classes are mainly determined by genetic background. During exposure to stress, barley tends to accumulate specific sterols, suggesting that sterols have a prominent role in plants' resistance. It was also found out that increased synthesis of stigmasterol during heat/drought may be associated with inbred stress resistance of barley.

The characteristics of lipidome changes in examined plant material allowed the extraction of interesting barley genotypes, i.a. MCam53 and CamB1 to be chosen for in-depth examination. In fact, additional research on other treatments such as high light as well as other parameters (e.g. lipid peroxidation products) could be investigated and monitored. Our results, supported by previous data on MCam53, permit the suggestion that this RIL may be successful for barley breeding and development of improved cultivars under changing climates.

Since the identification of lipid-binding proteins is a crucial aspect for deciphering components of lipid signaling cascades, it would be of interest to characterize the *LTP2* gene expression profile in selected plant material and to integrate these results with lipidome data in the future. The relation of brassinosteroids level in extreme genotypes (i.e. Maresi and MPW14/19) with respect to campesterol content (as the BS precursor) is another aspect that would be worthwhile examining.

Authors' contribution

AK, KM conception and design of the study; AK funding acquisition, project coordination; MK, PO plant breeding and stresses application; MK, PO, KM, AK sampling of plant material; VC methodology validation, statistics and computations; AK, KM, VC samples preparation, laboratory work and analyses; MTR-E supervision of the laboratory work and analyses; AK, VC, KM, MTR-E data analysis and interpretation, manuscript writing, revision and editing; AK, VC, PO, MK, MTR-E and KM contributed to the final version of the manuscript.Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.033.

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