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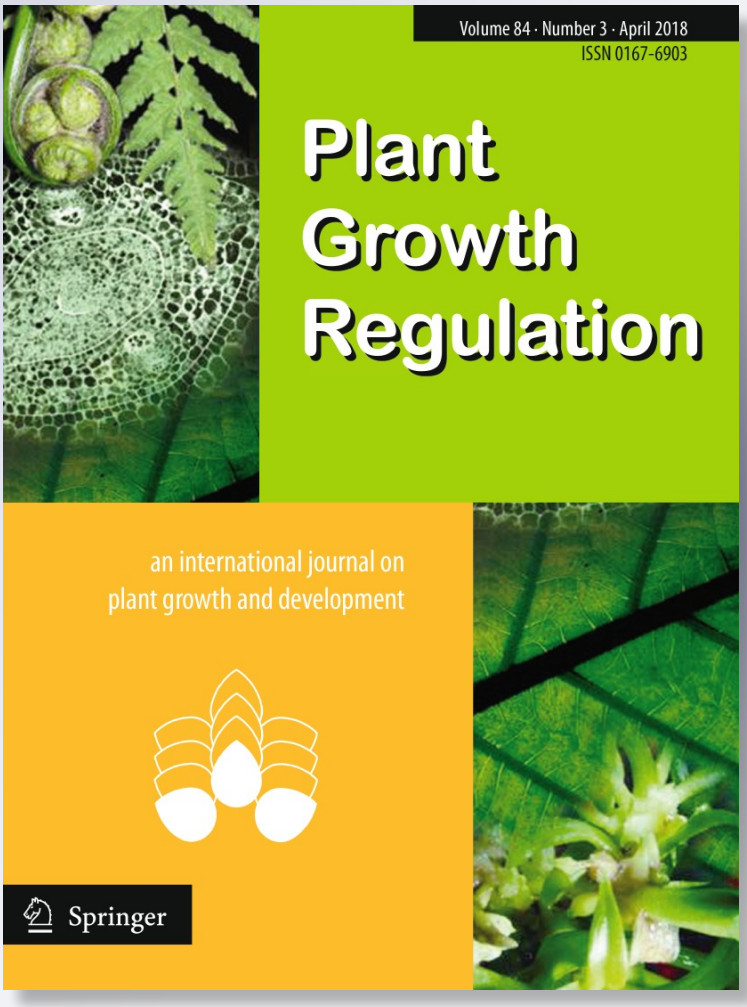

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ORIGINAL PAPER

Metabolomics of groundnut (*Arachis hypogaea* L.) genotypes under varying temperature regimes

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Abstract

Various metabolites were analyzed in groundnut genotypes grown under varying temperature regimes (based on date of sowing). Four contrasting groundnut genotypes viz. ICGS44 (high-temperature tolerant), AK159 and GG7 (moderately-high-temperature tolerant), and DRG1 (high-temperature sensitive) were grown at three different temperature regimes i.e., low (early date of sowing), normal (normal date of sowing) and high temperature (late date of sowing) under field conditions. Untargeted metabolomic analysis of leaf tissue was performed by GC–MS, while targeted metabolite profiling was carried out by HPLC (polyamines) and UPLC-MS/MS (phenolics) at both the pegging and pod filling stages. Untargeted metabolomic profiling revealed exclusive expression/induction of beta-D-galactofuranoside, L-threonine, hexopyranose, D-glucopyranose, stearic acid, 4-ketoglucose, D-gulose, 2-O-glycerol-alpha-d-galactopyranoside and serine in ICGS44 during the pegging stage under high-temperature conditions. During the pod filling stage at higher temperature, alpha-D-galactoside, dodecanedioic acid, 1-nonadecene, 1-tetradecene and beta-D-galactofuranose were found to be higher in both ICGS44 and GG7. Moreover, almost all the metabolites detected by GC–MS were found to be higher in GG7, except beta-D-galactopyranoside, beta-D-glucopyranose, inositol and palmitic acid. Accumulation of putrescine was observed to be higher during low-temperature stress, while agmatine showed constitutive expression in all the genotypes, irrespective of temperature regime and crop growth stage. Interestingly, spermidine was observed only in the high-temperature tolerant genotype ICGS44. In our study, we found a higher accumulation of cinnamic acid, caffeic acid, salicylic acid and vanillic acid in ICGS44 compared to that of other genotypes at the pegging stage, whereas catechin and epicatechin were found during the pod filling stage in response to high-temperature stress, suggesting their probable roles in heat-stress tolerance in groundnut.

Keywords Groundnut · High-temperature · Low-temperature · Metabolomic · Phenolics · Polyamines · Sugar

Abbreviations

dH2O	Distilled water
GC–MS	Gas chromatography–mass spectrometry
GSH	Reduced glutathione
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
MDA	Malondialdehyde
MSTFA	*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide
MUFA	Mono unsaturated fatty acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PLS-DA	Partial least squares discriminant analysis
PUFA	Poly unsaturated fatty acid
ROS	Reactive oxygen species

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TQ	Tandem quadrupole
UPLC-MS/MS	Ultra pressure liquid chromatography-tandem mass spectrometry

Introduction

Groundnut (*Arachis hypogaea* L.), a nutritious oilseed legume, is the sixth most important source of edible oil and the third most important source of vegetable protein in the world. High-temperature stress has a major influence on plant development and reproduction phase. Reproductive processes, particularly those of microsporogenesis and megasporogenesis, anthesis, pollination, pollen tube growth, fertilization and early embryo development are all highly susceptible to high-temperature stress. Exposure to temperatures > 33 °C for the first half of the day (6 h after anthesis) decreases pollen viability and thus seed-set in groundnut. High-temperature stress can also decrease biomass production, particularly if the stress is high enough to cause a decrease in photosynthesis (Djanaguiraman and Vara Prasad 2014). The effect of low temperatures above freezing is also an important stress factor limiting crop productivity (Mahajan and Tuteja 2005). The cessation of growth resulting from cold stress reduces the capacity for energy utilization, causing a feedback inhibition of photosynthesis and production of ROS. Membrane composition and fluidity are the key changes involved in low temperature perception. Indeed, at low temperatures, cell plasma membranes undergo phase transition in which the fluidity of the membrane is reduced to form a solid gel, which is used by plant cells to sense cold stress (Arbona et al. 2013). Low temperatures have also been recognized as an important facilitator of decreases in nutrient (Boron) absorption rates (An et al. 2012). It has been recognized that temperature acclimation results from a complex process involving a number of physiological and biochemical changes, including changes in membrane structure and function, tissue water content, gene expression, protein, lipid, and primary and secondary metabolite composition (Kaplan et al. 2004).

Various cellular metabolites are altered during drought and heat stress, including soluble sugars, amino acids, organic acids, phenolics, lipids, cofactors, nucleotides, peptides and secondary metabolites (Das et al. 2017). These metabolites can function as osmolytes to modify cellular water relations; as compatible solutes to stabilize enzymes, membranes and other cellular components; as chelating agents to neutralize or sequester potentially toxic levels of metals and inorganic ions; as antioxidants to scavenge reactive oxygen species; and as energy sources (Guy et al. 2008). In the present investigation we performed profiling of untargeted metabolites using gas chromatography–mass spectrometry (GC–MS) along with targeted metabolites, i.e., polyamines and phenolics, to identify specific metabolite(s) that exhibit temperature-specific responses during high- and low-temperature stress in distinct groundnut genotypes.

Materials and methods

Plant material, seed sowing, and stress condition used in the experiments and sample collection

Based on preliminary screening at the ICAR-Directorate of Groundnut Research, Junagadh, India, high-temperature tolerant groundnut genotype ICGS44, two moderately high-temperature tolerant genotypes GG7 and AK159, as well as high-temperature sensitive genotype DRG1, were selected for the present research. Groundnut genotypes ICGS44, GG7, AK159 and DRG1 were grown in the field during the summer of 2015 at ICAR- Directorate of Groundnut Research, Junagadh on three different dates (20 day interval between each sowing) in accordance with the following schedule: (i) January 20th, 2015 (early sowing), (ii) February 10th, 2015 (normal sowing), (iii) March 2nd, 2015 (late sowing). The maximum and minimum mean temperatures for the month of January were 29.2 and 11.6 °C, respectively, for February 32.0 and 14.6 °C, respectively, and for March 35.7 and 16.6 °C, respectively. Twenty seeds of each genotype were grown in three replications at each stage. Sampling was carried out at pegging and pod filling stages. The upper third leaves were collected from five plants grown in each replication on (i) 12th March, 2015, i.e., at pegging stage for early sowing (ii) 31st March, 2015, i.e., pod filling stage of early sowing and pegging stage of normal date of sowing & late sowing and (iii) 16th April, 2015, i.e., pod filling stage of both normal sowing and late sowing genotypes. The maximum and minimum mean temperatures for the month of March were 35.7 and 16.6 °C, respectively, and for April 39.3 and 22.9 °C, respectively. Samples were immediately snap-chilled in liquid nitrogen and stored at − 80 °C until metabolite extraction.

Plant biomass

Five plants were sampled randomly from each of the three replications of three different dates of sowing treatments during pegging stage. Plants were oven-dried at 65 °C until a constant weight was obtained. The average dry weight of five plants were calculated and expressed as per plant basis (g^{-1} plant ± SE).

Untargeted metabolomic profile by GC-MS

Extraction and derivatization of metabolites

Untargeted metabolomic (whole metabolomic) study was performed using GC–MS. Metabolites were extracted as described by Lisec et al. (2006) with minor modifications. Leaf tissues (200 mg) were homogenized with pre-chilled mortar-pestle in 3 ml of 100% HPLC grade methanol (pre-cooled at − 20 °C). Then 100 µl of ribitol (0.2 mg ml^{-1} stock in dH_2O) was added as an internal quantitative standard and vortexed for 10 s. The mixture was shaken for 10 min at 70 °C in a water-bath at 950 rpm and centrifuged for 10 min at 11,000 *g*. The supernatant was transferred to a Schott GL14 glass vial and 1.5 ml of chloroform (− 20 °C) was added. After that 3.0 ml of dH_2O (4 °C) was added and vortexed for 10 s. Again, the mixture was centrifuged for 15 min at 2200 *g* and the upper phase (polar) and lower phase (non-polar) phase were transferred into a separate test tube. Both polar and nonpolar phase were dried in a nitrogen stream.

Extracted metabolites were derivatized as described by Sanimah et al. (2013) with minor modifications. The dried extracts were re-dissolved in 50 µl of pyridine and sonicated for 10 min. Then, 100 µl of methoxyamine HCL (20 mg ml^{-1} in pyridine) was added and vortexed for 30 s. The mixtures were then sonicated again for 5 min and incubated with constant agitation for 90 min at 37 °C. The trimethylsilylation (TMS) step was performed by adding 250 µl *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) to the extracts and vortexed for 30 s. Mixtures were incubated for 1 h at 37 °C for derivatization.

Metabolomic analysis by GC-MS

For GC–MS analysis, 1 µl of derivatized extract was injected into a DB-17MS capillary (30 m × 0.25 mm). The inlet temperature was set at 280 °C. After a solvent delay for 5 min, initial GC oven temperature was set at 100 °C; after injection for 1 min, the GC oven temperature was raised to 290 °C. The injection temperature was set to 280 °C and ion source temperature was 230 °C. Helium was used as the carrier gas with a constant flow rate set at 1 ml/min. The measurement was performed with electron impact ionization (70 eV) in the full scan mode (m/z from 50 to 700). Metabolites were putatively identified by matching their mass spectra to spectra in NIST 14 library (National Institute of Standards and Technology, Gaithersburg, MD, USA). Pre-processing of total ion chromatograms (TIC) such as baseline correction, alignment, peak picking, and integration were performed using the ACD/Spec Manager v.12.00 (Advanced Chemistry Development, Inc., ACD/Labs, Toronto, Canada). CSV comma delimited files were created for data analysis.

Targeted metabolite profiles

Extraction, derivatization and estimation of polyamines using HPLC

Leaf tissues were extracted in 5% cold perchloric acid ($HClO_4$) at a ratio of about 200 mg/2 ml $HClO_4$. After extraction for 1 h in an ice bath, samples were pelleted at 15,000 rpm for 20 min and the supernatant phase, containing the 'free polyamine fraction' was stored frozen at − 20 °C in eppendorf centrifuge tubes (Mhaske et al. 2013).

Benzoylation and HPLC analysis

Polyamines were analyzed through HPLC as described by Flores and Galston (1982) with minor modifications. Plant extracts were benzoylated along with standards. One ml 2 N NaOH was mixed with 250–500 µl of $HClO_4$ extract followed by addition of 10 µl benzoyl chloride, vortexing for 10 s. It was incubated for 20 min at room temperature. To this, 2.0 ml saturated NaCl was added and mixed by vortexing. To this, 2.0 ml diethyl ether was added and centrifuged at 3000 rpm for 5 min. Benzoyl polyamines were extracted in ether phase so, 1.0 ml of the ether phase was collected, dried in a vacuum concentrator without heating, and was redissolved in 100 µl absolute methanol (HPLC grade). Standards were treated in a similar way, with up to 100 ppm of each polyamine in the reaction mixture. The benzoylated samples were stored at − 20 °C. The solvent system consisted of methanol:water run isocratically at 60% at the flow rate of 1 ml min^{-1}. The benzoylated extracts were eluted at room temperature through a 4.6 × 250 mm, 5 µm particle size reverse- phases (C18) column and detected at 254 nm. The quantity of individual polyamines was calculated based on area and concentration of standards.

Extraction and estimation of phenolics using UPLC-MS/MS

Phenolic compounds were extracted as described by Jadhav et al. (2013) with minor modifications. Dried leaves (500 mg) of groundnut were grounded and extracted with 2 ml of an 80% methanol solution (v/v) at room temperature for 20 min and the resulting solution was filtered and dried in a vacuum concentrator without heating. Then, the residues were redissolved in 100 µl of absolute methanol and 10 µl from it was injected to UPLC-MS/MS. Phenolic compounds were analyzed through UPLC-MS/MS as described by Xia et al. (2011) with minor modifications. Sixteen standards (cinnamic acid, caffeic acid, salicylic acid, gallic acid, ferulic acid, quercetin, catechol, chlorogenic acid, coumaric acid, syringic acid, kaempferol, vanillic acid, catechin, epicatechin, epigallocatechin and resveratrol) were used in

present study. Each standard was dissolved in 80% methanol and separated individually then a mixture of standards (500 µg ml^{-1} of each phenolic) was run and identified based on retention time and mass.

UPLC was performed on a Waters Acquity UPLC system (Waters, Milford, USA), equipped with a binary solvent delivery system and an autosampler. The chromatographic separation was performed at 35 °C on a Waters Acquity BEH C_{18} column (100 mm × 2.1 mm, i.d., 1.7 µm). Separation was performed with a binary mobile phase at 0.4 ml/min during the analysis and the sample injection volume was 10 µl. The optimized separation conditions were solvent (A) methanol and (B) 1% (volume fraction) acetic acid in water as gradient run (Supplementary Table S1).

The UPLC instrument was coupled to a TQ mass spectrometer (Aquity). A MS system equipped with electrospray ionization (ESI) source operated in negative-ion mode and a multiple reaction monitoring (MRM) scan mode. ESI ionization conditions were source temperature 150 °C, negative ionization mode, source voltage − 3.2 kV. High-purity nitrogen (> 99.999%) was used as curtain and auxiliary gas. The quantity of individual phenolics was calculated based on area and concentration of standards.

Data processing and statistical analysis

Data processing and statistical analysis of untargeted metabolites was carried out using MetaboAnalyst 3.0 (Xia et al. 2015), an online statistical package and the data were normalized with respect to the internal standard ribitol. Data processing and time series analysis of polyamines and phenolic compounds was carried out with same statistical package. Data were normalized with pareto scaling for both targeted and untargeted metabolomic analysis.

Results

Biomass

The biomass accumulation pattern of groundnut genotypes at pegging stage during low, normal and high temperature (based on different dates of sowing) was clearly distinguishable in the present study (Supplementary Table S2). The highest biomass was recorded in low-temperature grown plants, which may be attributed to longer crop growth duration. Biomass content was reduced by 28–47% due to the increase in temperature regime from low to high temperature during crop growth period. Maximum reduction in biomass was observed in the genotype AK159 (46.79%) followed by DRG1 (39.30%), GG7 (39.08%) and ICGS44 (28.44%). Thus, our results clearly indicated that genotype ICGS44 was the least affected by high temperature stress on biomass accumulation.

Untargeted metabolites

A total of 67 and 75 metabolites of known structure comprising sugars, sugar alcohols, fatty acids, amino acids, organic acids, phenols and sterols were identified at pegging and pod filling stages, respectively, in leaf extracts of groundnut using the NIST library. Sugars, fatty acids and organic acids were predominant in leaf extract compared to other metabolites.

Distinguished metabolite patterns in groundnut leaves were observed at different temperatures during pegging stage. Highly abundant metabolites at different temperature regimes were identified by PLS-DA using variable importance in projection (VIP). Inositol, palmitic acid, stearic acid, stigmasterol and D-glucopyranose showed greater accumulations at high temperature, while beta-D-galactofuranoside, butanal, malic acid, mannose, hexopyranose and 9,12,15-octadecatrien-1-ol showed higher accumulations at normal temperature. Galactose, tridecanol, myristic acid, docosane, 4-ketoglucose and linolenic acid were observed to be higher at low temperature during the pegging stage (Supplementary Fig. S1). At the pod filling stage, higher accumulations of beta-D-galactopyranoside and beta-D-glucopyranose were observed during normal temperature, whereas higher accumulations of glucitol, xylulose, stearic acid, myo-inositol and mannose were observed during higher temperature (Supplementary Fig. S2). These results imply that comparatively higher number of metabolites was accumulated in low-temperature grown plants at the pegging stage, while at the pod filling stage, most of the metabolites were synthesized in high-temperature grown plants.

A heat map analysis of 67 metabolites showed that butanedioic acid, pentitol, stigma sterol, beta-D-galactofuranoside, 2-monostearin, palmitic acid, 2-keto-d-gluconic acid, L-threonine, hexopyranose, D-glucopyranose, stearic acid, 4-ketoglucose, gulose, 2-O-glycerol-alpha-d-galactopyranoside, D-mannitol, linolenic acid, serine and propanoic acid were synthesized more in ICGS44 than DRG1 under a high-temperature treatment at the pegging stage (Fig. 1). Additionally, most of the above compounds were accumulated in GG7 at high-temperature conditions, but their concentrations were less compared to those of ICGS44. Among the metabolites, beta-D-galactofuranoside, hexopyranose, 4-ketoglucose, gulose, D-mannitol, maltose, galactose and serine were exclusively observed in ICGS44 at high temperatures; thus, these metabolites can be used as biomarkers for high-temperature stress tolerance. At normal temperature, almost all the above compounds and hexopyranose, 9-tricosene, 3-hydroxy glutaric acid and 2-butenedioic acid were found to be higher in ICGS44 and hexopyranose, butanal,

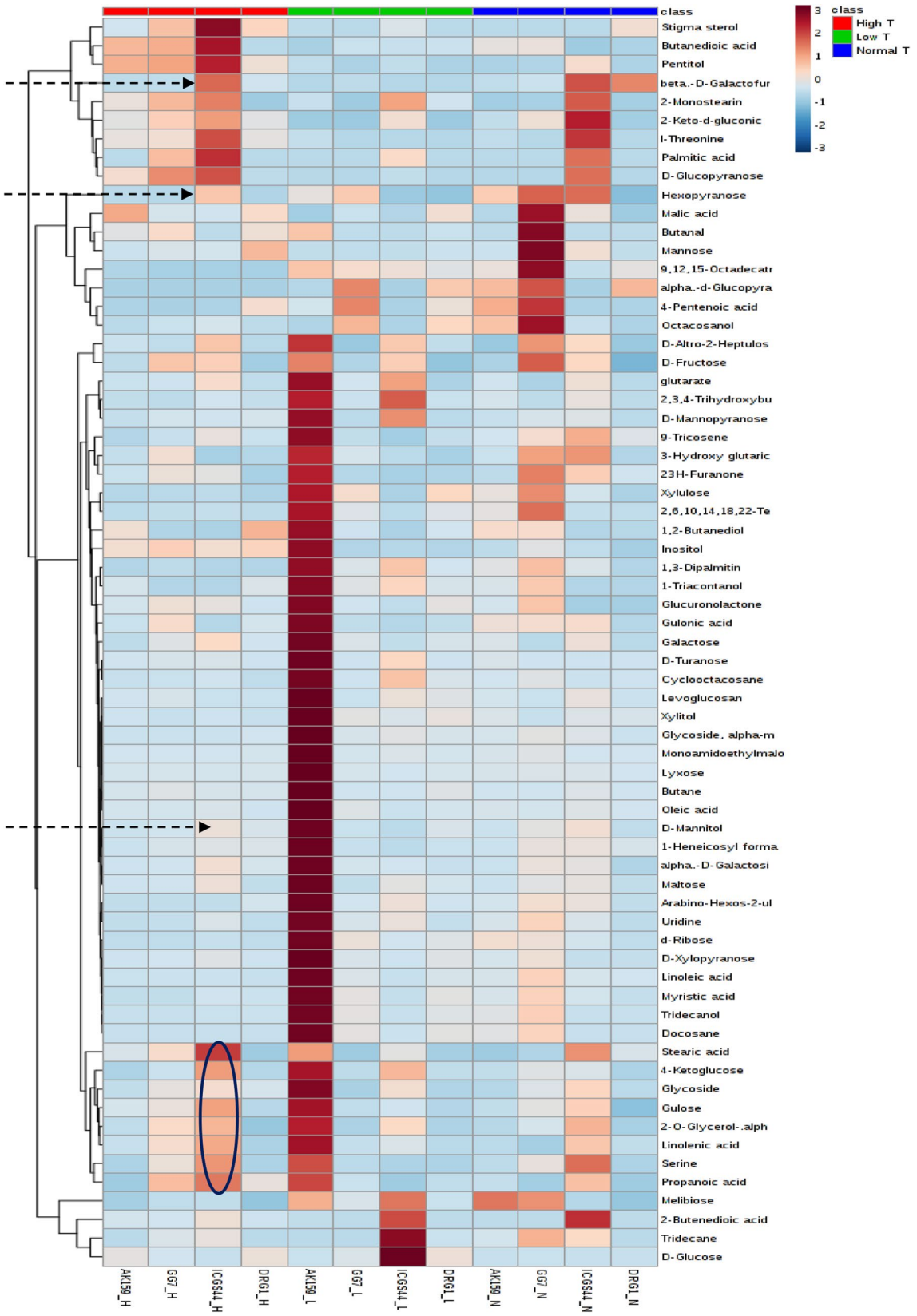

Fig. 1 Heat map analysis shows intensity of metabolites in groundnut genotypes during three different temperatures at the pegging stage. Where: H, N and L denoted higher-temperature, normal- temperature and low-temperature, respectively

malic acid, mannose, 9,12,15-octadecatrien-1-ol, alpha-d-glucopyranoside, 4-pentenoic acid, D-altro-2-heptulose, D-fructose, octacosanol, 2,6,10,14,18,22-tetracosahexaene, 2(3H)-furanone, xylulose, tridecane and melibiose were observed to be higher in GG7. However, D-glucose, tridecane, 2-butenedioic acid, melibiose, 4-ketoglucose, D-mannopyranose, 2,3,4-trihydroxybutyric acid, glutarate, D-fructose, D-altro-2-heptulose and 2-monostearin were detected at higher levels in ICGS44 while octacosanol, alpha-d-glucopyranoside and 4-pentenoic acid were more highly accumulated in GG7 at low temperature. The level of most of the metabolites remained lower in DRG1 at higher and lower temperatures (Fig. 1).

A total of 75 metabolites were detected at the pod filling stage. A heat map analysis of the metabolites showed that tridecanol, D-glucose and 1-nonadecene were accumulated more in AK159, whereas alpha-D-galactoside, dodecanedioic acid, 1-nonadecene, 1-tetradecene and beta-D-galactofuranose were more highly accumulated in ICGS44 during high-temperature treatment. Most of the metabolites, except beta-D-galactopyranoside, beta-D-glucopyranose, inositol and palmitic acid were observed in abundance in GG7 during the higher temperature condition, while low levels of all the metabolites were observed in DRG1 at the high-temperature condition. At low temperature, D-galactose and xylonic acid were synthesized at higher levels in AK159 while 4-D-ribopyranose, malic acid and linolenic acid were synthesized at higher levels in GG7. D-galactose, xylonic acid and 3-hexadecene were produced at higher levels in ICGS44, while linolenic, linoleic, oleic and palmitic acid along with inositol and gluconic acid were detected in appreciable amounts in DRG1 under the low-temperature regime. At normal temperature, AK159 had higher amounts of 2-monostearin and beta-D-galactofuranose, while GG7 had higher amounts of glycine, beta-D-galactopyranoside, beta-D-glucopyranose, L-threonine, fructose, 4-ketoglucose and melibiose. At the normal-temperature regime, gulonic acid, D-galactose, xylonic acid, arabino-hexos-2-ulose were synthesized at higher levels in ICGS44, while xylonic acid, D-glucose, arabino-hexos-2-ulose were synthesized at higher levels in DRG1 (Fig. 2).

Based on dendrogram analysis at the pegging stage, genotype ICGS44 was clustered in a separate sub-cluster at all three temperature regimes, while genotypes DRG1 and AK159 shared the same sub-sub-cluster at high temperature, and GG7 occupied a separate sub-sub-cluster. AK159 showed a completely different pattern of metabolites at low temperature and thus was grouped alone on cluster-I. Interestingly, genotype ICGS44 showed similar metabolites during high as well as normal temperature and shared the same sub-cluster with few differences in metabolite level at low temperature. DRG1 (temperature sensitive) shared similarity with AK159 at both high- and normal-temperature regimes (Fig. 3). A dendrogram analysis at the pod filling stage suggested that all the genotypes showed almost similar patterns of metabolic profiling at low, normal and high temperatures, except GG7 at high temperature (Fig. 4). Metabolomics data were further analyzed using random forest to identify potential outliers (genotypes) in different temperature regimes. At the pegging stage, two outliers were identified, i.e., ICGS44 at high temperature and AK159 at low temperature (Supplementary Fig S3). At the pod filling stage, three outliers were identified, i.e., GG7 at high temperature, DRG1 at low temperature and GG7 at normal temperature (Supplementary Fig S4).

Targeted metabolites

Polyamines

Five polyamines, i.e., putrescine, cadaverine, agmatine, spermidine and spermine were identified and quantified in leaf extracts at both the pegging and pod filling stages of groundnut using HPLC (Supplementary Table S3). A heat map analysis of polyamines (Fig. 5) suggested that more putrescine was produced in AK159, while spermidine was higher in GG7 and in DRG1 during low temperature at the pegging stage. However, during high temperature at the pegging and pod filling stages, spermidine was observed only in the high-temperature tolerant genotype ICGS44. Higher accumulation of spermine was detected in GG7 during normal temperature, while cadaverine was higher in DRG1 at higher temperature. Agmatine was constitutively synthesized in all the genotypes during all temperatures at the pegging and pod filling stage.

Phenolic compounds

Sixteen phenolics, namely, cinnamic acid, caffeic acid, salicylic acid, gallic acid, ferulic acid, quercetin, catechol, chlorogenic acid, coumaric acid, syringic acid, kaempferol, vanillic acid, catechin, epicatechin, epigallocatechin and resveratrol were identified and quantified in leaf extracts at the pegging and pod filling stages of groundnut using LC-MS/MS (Supplementary Table S4 A, B). A heat map analysis revealed that temperature-specific biosynthesis of phenolics was detected in all the four genotypes, particularly at low-temperature stress during pegging stage (Fig. 6). During low temperature at the pegging stage, coumaric acid, caffeic acid, gallic acid and salicylic acid were abundantly accumulated in all genotypes. Vanillic acid content was found to be higher in ICGS44 and DRG1, whereas resveratrol was found to be abundant in GG7, ICGS44 and DRG1. Caffeic acid and quercetin were synthesized at higher levels in AK159 and GG7 during low-temperature conditions. Interestingly, salicylic acid was observed to be higher in all genotypes

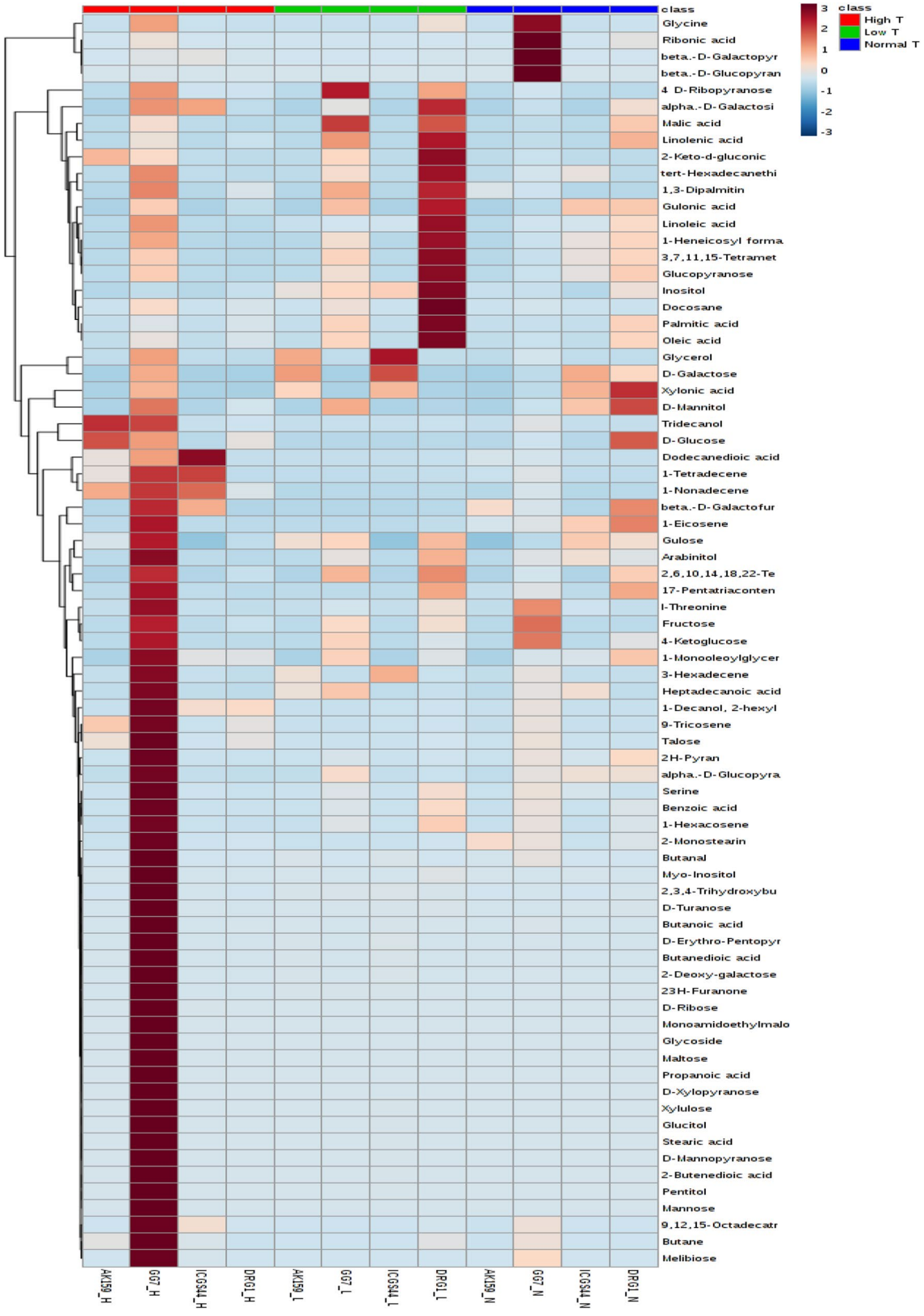

Fig. 2 Heat map analysis shows intensity of metabolites in groundnut genotypes during three different temperatures at the pod filling stage. Where: H, N and L denoted higher-temperature, normal- temperature and low-temperature, respectively

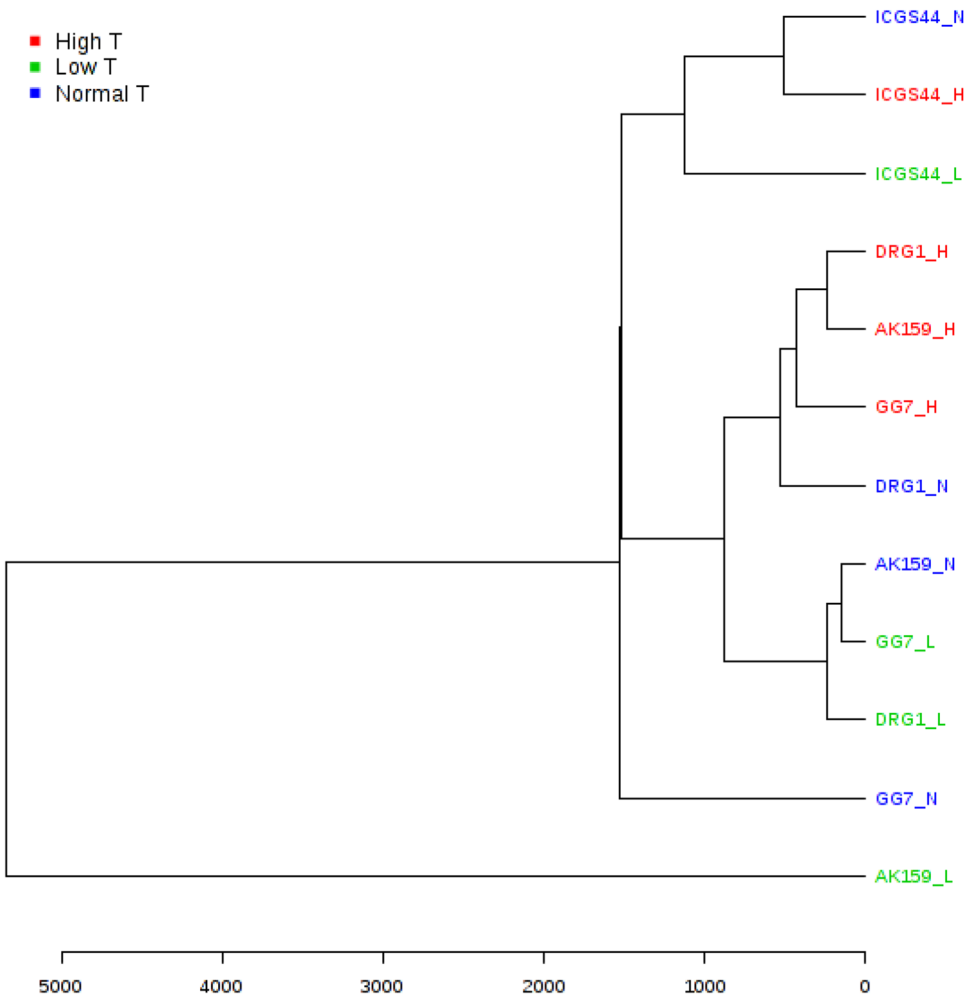

Fig. 3 Clustring pattern of groundnut genotypes at the pegging stage. Where: H, N and L denoted higher- temperature, normal-temperature and low-temperature, respectively

under the low-temperature regime. However, during high-temperature stress at the pegging stage, the accumulation of cinnamic acid, caffeic acid, salicylic acid and vanillic acid were more pronounced in ICGS44 compared to that of the rest of the genotypes, while at pod filling stage, contents of catechin and epicatechin were highest in ICGS44.

Discussion

Untargeted metabolomics

Different sugars, sugar alcohols, amino acids, organic acids and fatty acids were synthesized at both the stages during different temperature regimes. Accumulations of these metabolites varied in different genotypes and under different temperature conditions. A higher accumulation of soluble sugars (beta-D-galactofuranoside, hexopyranose, D-glucopyranose) and osmoprotectants (D-mannitol and pentitol) in ICGS44 may have played a crucial role in regulating osmotic adjustment and protecting various cellular structures from temperature stress by maintaining the cell-water balance and membrane stability and by buffering the cellular redox potential. Higher carbohydrate availability during heat stress was found to be an important physiological trait associated with stress tolerance and acclimation processes (Liu and Huang 2000). They can also act as antioxidants and play a key role in cellular defense functions; however, at lower concentrations, they act as signaling molecules (Sugio et al. 2009). In the present study, we found comparatively higher sugar accumulation during low temperature at the pegging stage. Many important metabolites viz., galactose, tridecanol, myristic acid, docosane and 4-ketoglucose accumulated in AK159 at the pegging stage under low temperature conditions. This might be an important acclimation process for low temperature tolerance in groundnut. Janska et al. (2009) reported that during cold stress, plant species acclimate by synthesizing soluble sugars, which act as cryoprotective molecules. These molecules stabilize both membrane phospholipids and proteins, including cytoplasmic proteins acting in conjunction with dehydrin proteins, cold-regulated proteins and heat-shock proteins. Cryoprotective solutes are

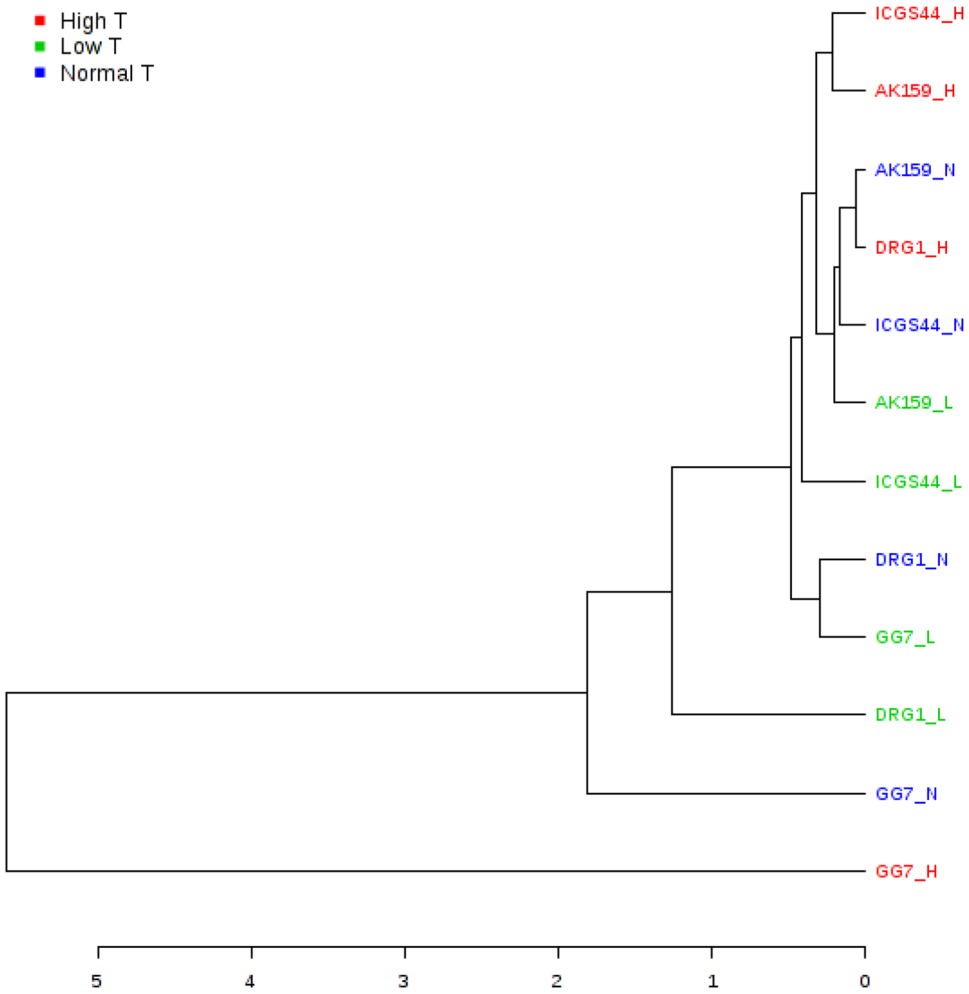

Fig. 4 Clustring pattern of groundnut genotypes at the pod filling stage. Where: H, N and L denoted higher-temperature, normal-temperature and low-temperature, respectively

also involved in the maintenance of hydrophobic interactions, the homeostasis of ions, the protection of the plasma membrane from adhesion of ice, the scavenging of ROS and the prevention of consequent damage to cells (Awasthi et al. 2015).

In the present study, as many as 67 metabolites were induced under high-temperature stress in the heat-tolerant genotype ICGS44 compared to the heat-sensitive genotype DRG1 at the pegging stage. These metabolites include many organic acids, amino acids, sugar alcohols and other carbohydrates (Fig. 1). Higher levels of organic acid were also reported in groundnut during heat stress, which was suggested to be an indicator of increased photochemical efficiency of groundnut seedlings during early stages of heat stress. Many of the amino acids also act as osmoprotectants, and higher level of these amino acids were proposed to be associated with improved membrane stability and tolerance during heat stress (Singh et al. 2016).

As an integral component of the cell membrane, fatty acids and their composition play an important role in maintaining the fluidity and integrity of the cellular membrane during various degrees of temperature stress (Buchanan et al. 2000). A greater share of saturated fatty acids in membrane lipids increases the lipid melting temperature and prevents a heat-induced increase in the membrane fluidity. Thus, increasing the saturation level of fatty acids appears to be critical for maintaining membrane stability and enhancing heat tolerance (Bita and Gerats 2013). We found higher accumulations of palmitic and stearic acid in ICGS44 under high-temperature stress. Moreover, a higher accumulation of linoleic and linolenic acid in AK159 during low temperature further confirmed the role of unsaturated fatty acids in maintaining membrane fluidity and low-temperature stress tolerance. Alteration in the membrane lipid composition by increased fatty acid unsaturation is an adaptation strategy of living cells to chilling temperatures (Sanghera et al. 2011). Singh et al. (2016) reported the highest saturated to unsaturated fatty acid ratio in the high-temperature tolerant Virginia genotype of groundnut. Upchurch (2008) suggested that plants capable of cold acclimation accumulate more polyunsaturated fatty acids during cold stress. The results from our present study showed fatty acids played important roles

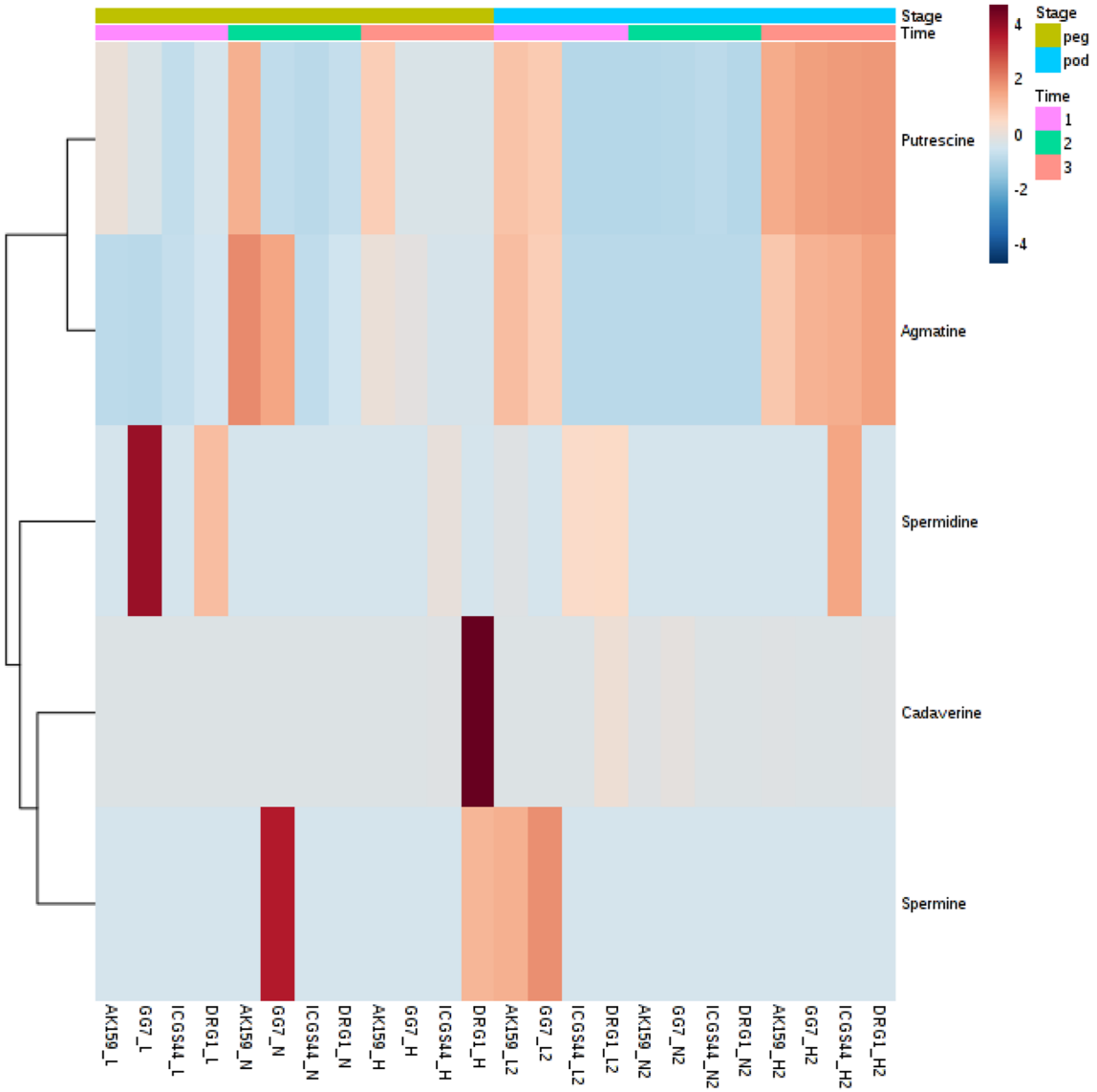

Fig. 5 Heat map analysis shows intensity of polyamines during three different temperatures at the pegging and pod filling stage. Where: H, N and L denoted higher-temperature, normal-temperature and low-temperature, respectively at pegging stage; and H2, N2 and L2 denoted higher- temperature, normal-temperature and low-temperature, respectively at pod filling stage

in high- as well as low-temperature acclimation processes in groundnut, thus helping to impart tolerance in respective genotypes, where characteristic fatty acids were synthesized under high and low temperature stress.

Targeted metabolites

Polyamines

Although we observed a low level of accumulation of spermidine in the heat-tolerant genotype ICGS44, its unique induction in only this genotype indicated its crucial role in high-temperature tolerance in groundnut. Spermidine, a tetravalent polyamine reported to effectively improve the total antioxidant ability of plants, enhanced the tolerance of the plants to high temperature stress (Todorova et al. 2015). It also improves the fluorescence properties of chlorophyll, hardening and increasing the resistance of the pigment-protein complexes to thermal damage, and enhancing the activity of PSII during a linear increase in temperature (Murkowski 2001). Spermidine also modulated carbohydrate and reduced glutathione levels that might be involved in the simultaneous induction of the antioxidant defense systems and endogenous hormone metabolism, thereby enhancing the heat and chilling stress tolerance in rice seedlings (Mostofa et al. 2014; Yan-hua et al. 2015). Protective effects

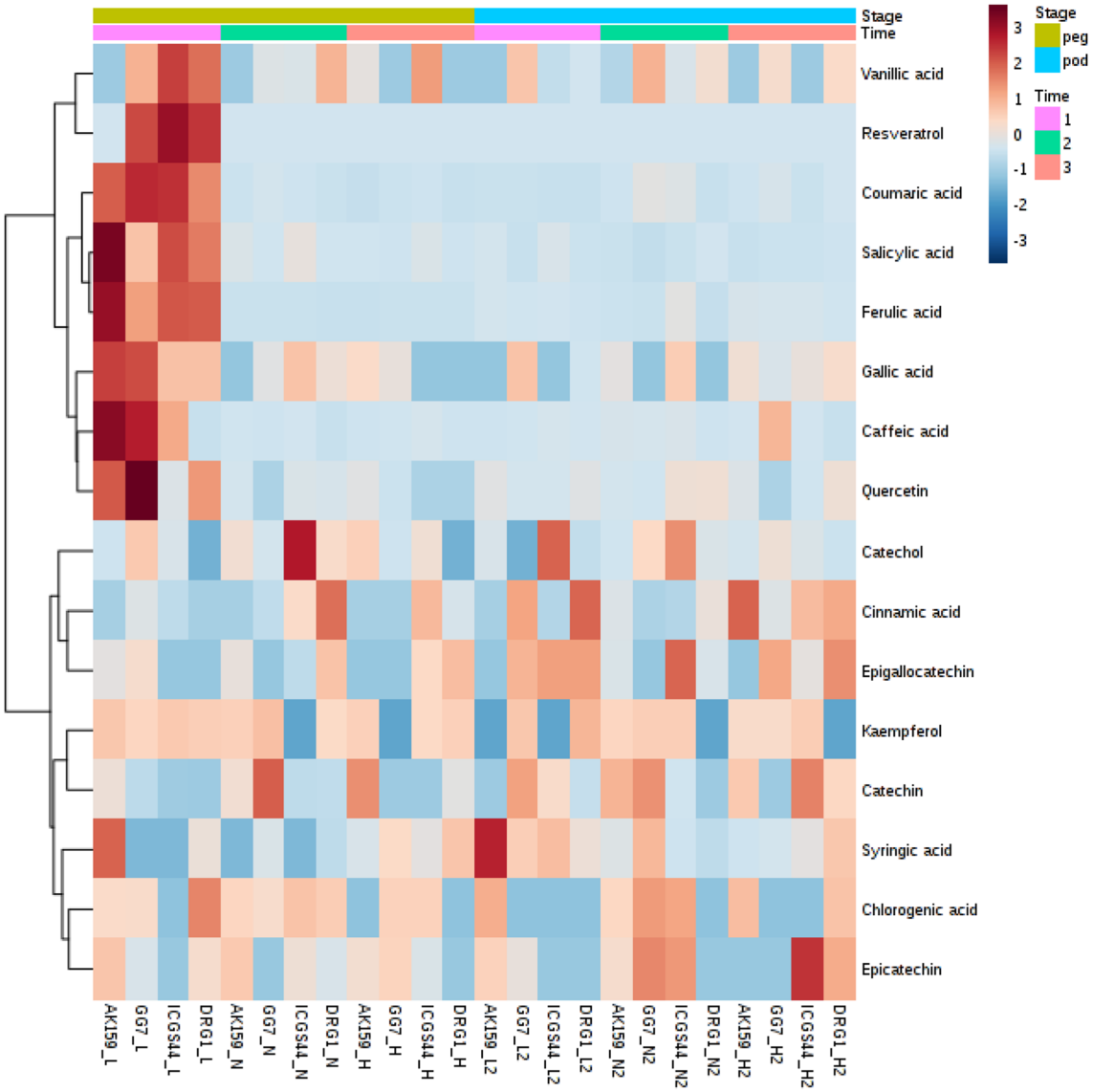

Fig. 6 Heat map analysis shows intensity of phenolics during three different temperatures and at pegging and pod filling stage. Where: H, N and L denoted higher-temperature, normal- temperature and low-temperature, respectively at pegging stage; and H2, N2 and L2 denoted higher-temperature, normal-temperature and low-temperature, respectively at pod filling stage

of spermidine are also reflected by its roles in maintaining osmoregulation, water status and improved mung bean seedling growth during low temperatures (Nahar et al. 2015). Spermidine may act as a cellular membrane protectant against chilling induced lipid peroxidation by diminishing electrolyte leakage and malondialdehyde (MDA) content (Alcazar et al. 2011).

Like spermidine, putrescine is another important polyamine that plays a crucial role, especially under low-temperature stress. It reduces cold-induced electrolyte leakage by improving the apoplastic antioxidant system and diminishing the MDA content (Zhang et al. 2009). Application of putrescine improved anthocyanin, flavonoids, phenolic compounds and peroxidase enzyme activity (antioxidant properties) in dragonhead plants grown under low temperature (Abavisani et al. 2013). In the present study, differential accumulation of both spermidine and putrescine was observed in thermo-tolerant and -sensitive groundnut genotypes, which indicated that these polyamines have broad functions in low- and high-temperature stress tolerance in the studied genotypes. No specific role in heat-stress tolerance of other polyamines viz. agmatine has been found in plants, but it was reported to play an osmoprotective role in drought stress (Arbona et al. 2013). In the present study, we did not observe much change in agmatine concentration under different temperature conditions, which might

be because agmatine is synthesized as an intermediate in putrescine biosynthesis from arginine. Hence, it might not have been detected, as it was completely converted to putrescine during this biosynthetic process.

Phenolics

In the present study, we found considerable changes in the phenolics composition in groundnut leaves grown under different temperature regimes. Higher accumulation of vanillic acid, resveratrol, coumaric acid, caffeic acid and salicylic acid at lower-temperature conditions was observed in all the studied groundnut genotypes. Such low-temperature induced phenolics production perhaps activated the antioxidant enzyme-mediated defense response in groundnut undergoing chilling stress (Koc et al. 2010). Moreover, it is known that low temperatures promote the accumulation of endogenous free salicylic acid in different crops (Xia et al. 2009; reviewed by; Miura and Tada 2014), suggesting that salicylic acid is involved in the regulation of low-temperature responses. Furthermore, stress-induced phenolics might be subsequently incorporated into the cell wall either as lignin or suberin, resulting in lignification and suberization, respectively, which are reported to enhance cold-temperature tolerance in plants (Ramakrishna and Ravishankar 2011).

Unlike the low temperature condition, accumulation of phenolics was very low during high temperature stress. However, the contents of cinnamic acid, caffeic acid, salicylic acid, ferulic acid and coumaric acid were higher in ICGS44 than that of other genotypes during the pegging stage, while catechin and epicatechin were higher during the pod filling stage. Such high-temperature stress-induced accumulation of phenolics was reported in a number of crops, including tomato and water melon, which could be one of the acclimation mechanisms of plants against thermal stress (Rivero et al. 2001). The higher induction of phenolics under heat stress also helped to maintain the osmotic potential of leaves by increasing water uptake and reducing transpiration loss. For example, foliar application of caffeic acid reduces electrolyte leakage from the cotyledon leaf and promotes the activities of α-amylase and β-amylase under high-temperature stress and thereby provides thermal protection in *Gossypium arboretum* plantlets (Thind and Barn 2012). Phenylpropanoid compounds such as anthocyanins, p-coumaric, caffeic, ferulic and sinapic acids are well-known ROS scavengers, which protect the cytoskeletal components of the cell and thus prevent heat-induced cell death (Commisso et al. 2016). In the present study, cinnamic acid, caffeic acid, salicylic acid, ferulic acid and coumaric acid were synthesized in less quantity during high temperature stress, but their differential induction suggested that they play an important role in heat-stress tolerance in groundnut.

Conclusion

In conclusion, at the low-temperature regime, most of the metabolites were synthesized at higher concentrations at the pegging stage, while at the higher-temperature regime, most of the metabolites were found to be accumulated at higher levels at the pod filling stage. Beta-D-galactofuranoside, L-threonine, hexopyranose, D-glucopyranose, stearic acid, 4-ketoglucose, gulose, 2-O-glycerol-alpha.-d-galactopyranoside and serine can be used as biomarkers for high-temperature stress tolerance. Spermidine, although produced in lower quantities, is thought to impart high-temperature stress tolerance to ICGS44. An appreciably higher accumulation of phenolics, i.e., vanillic acid, resveratrol, gallic acid, caffeic acid and salicylic acid during low temperature in all genotypes suggested a crucial role of phenolics in low-temperature stress tolerance in groundnut in general. A higher accumulation of cinnamic acid, caffeic acid, salicylic acid and vanillic acid in the heat-tolerant genotype ICGS44 during high-temperature stress at the pegging stage implied their critical role in heat-stress tolerance in groundnut.

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