

Supplementary information

Supplementary Table S1. The effect of tested variables on embryo-like structures formation in anther cultures of two winter triticale DH lines, after 5-azacytidine tiller treatment.

| Sources of variation | MS | p |
|--------------------------|------|-----|
| (1) Donor plant genotype | 5859 | *** |
| (2) Tiller treatment | 331 | *** |
| (1) × (2) | 421 | *** |

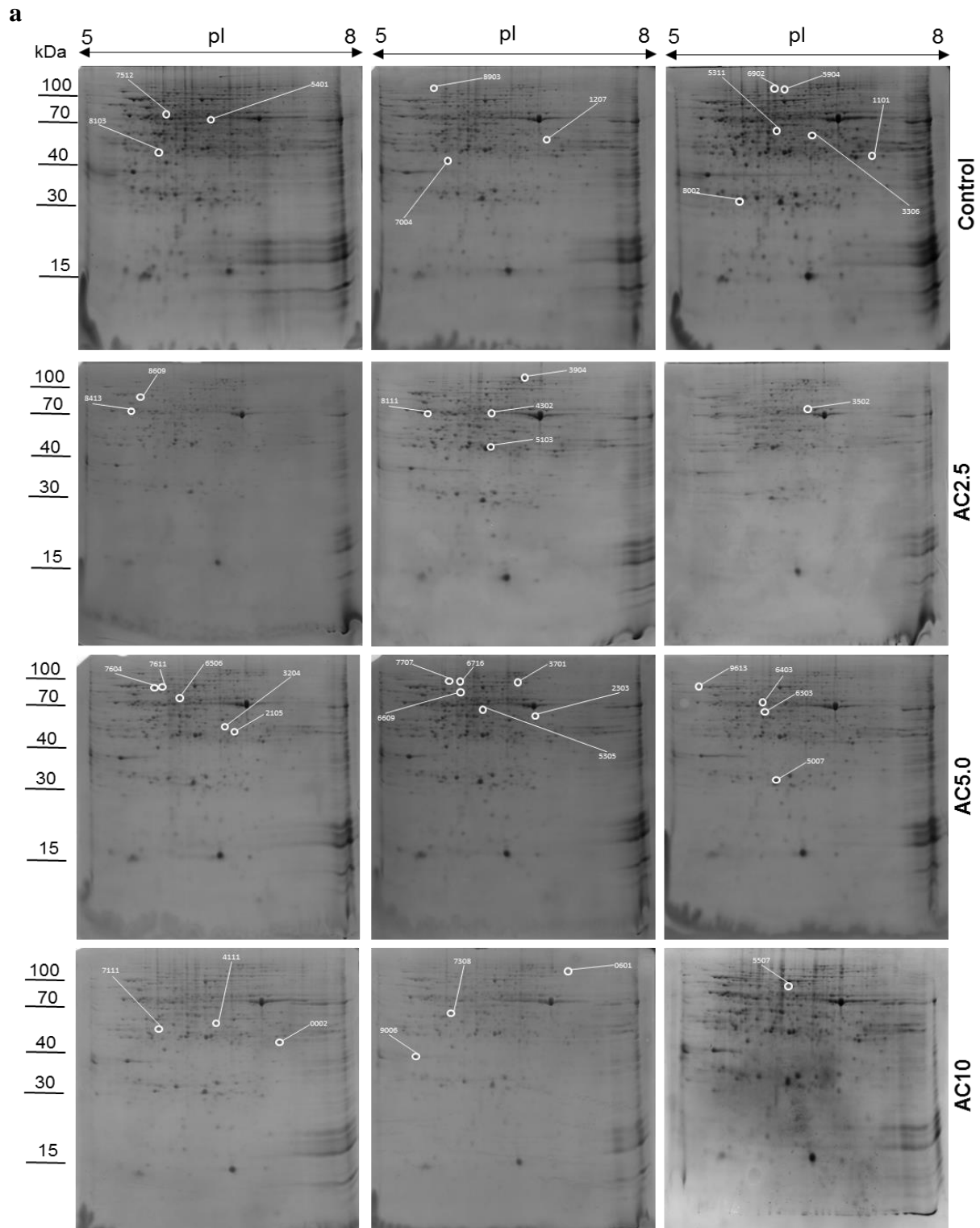
The sources of variance were as follows: two DH lines, three treatments, and interaction between DH line and treatment MS mean square, *** $p \leq 0.001$

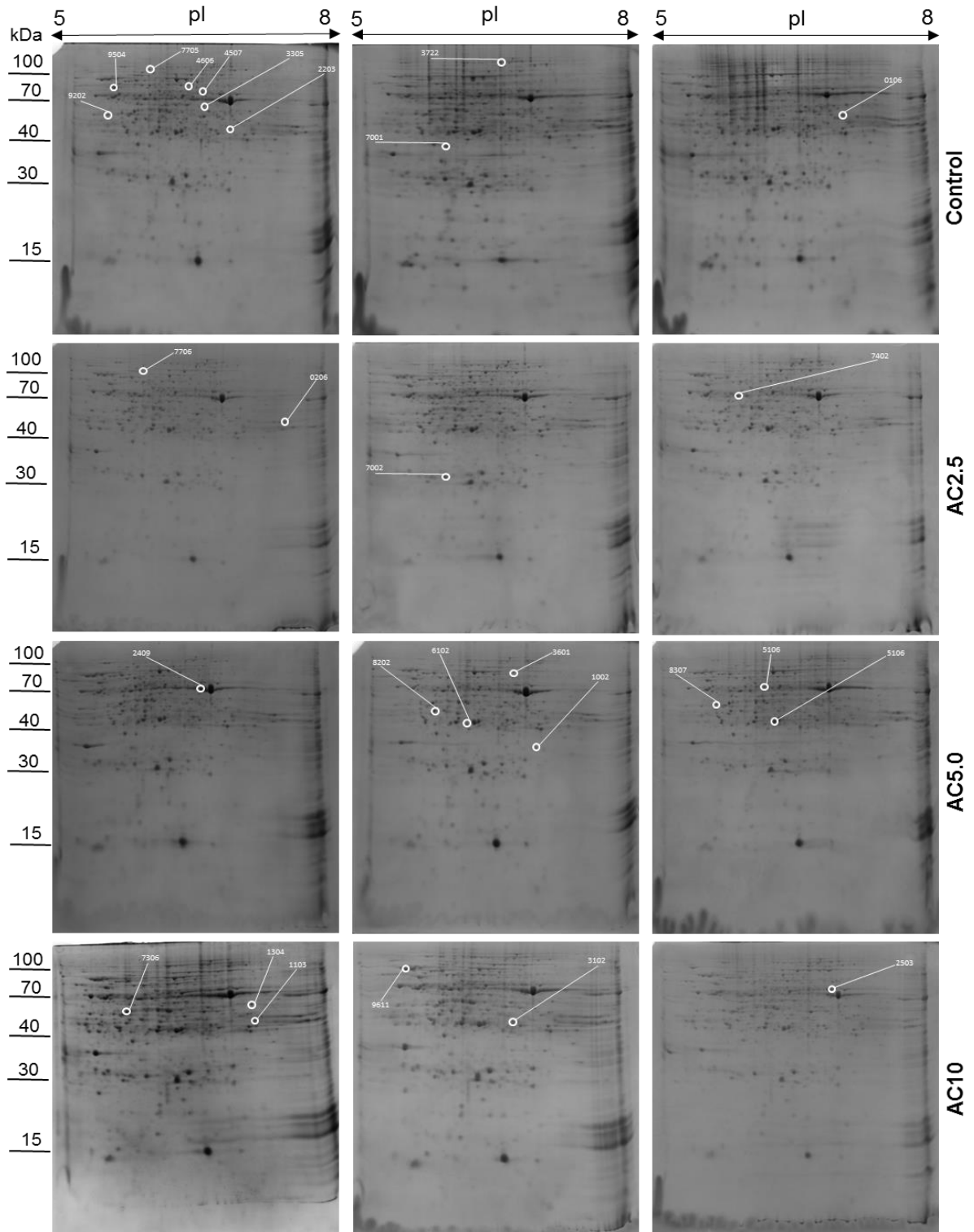
Supplementary Table S2. The total protein yield obtained from anthers of two winter triticale DH lines after various types of tiller treatments and the extraction based on phenol protocol . Data are the means from 3 biological replicates ± SE.

| DH line | Treatment | Anthers FW [g] | Protein content [µg/g FW] |
|---------|-----------|----------------|---------------------------|
| DH19 | Control | 3.9±0.17 | 893.1±181 ^{ab} |
| | AC2.5 | 1.4±0.06 | 2096.4±526 ^{bc} |
| | AC5.0 | 2.2±0.16 | 1507.7±423 ^{ab} |
| | AC10 | 2.0±0.04 | 667.6±8.5 ^a |
| DH28 | Control | 4.3±0.19 | 987.2±339 ^{ab} |
| | AC2.5 | 0.7±0.03 | 2196.3±652 ^c |
| | AC5.0 | 2.3±0.02 | 1031.0±63 ^{ab} |
| | AC10 | 2.8±0.06 | 479.1±84 ^a |

DH19 – recalcitrant DH line, DH28 – responsive DH line, Control – anthers isolated from tillers treated with low temperature (3 weeks at 4 °C), AC2.5, AC5.0, AC10 – anthers isolated from tillers treated with low temperature and 5-azacytidine at concentration of 2.5 µM, 5.0 µM and 10 µM respectively, FW – fresh weight of anthers.

Supplementary Figure S1. Electrophoregrams of triticale anther protein map after low temperature (3 weeks at 4 °C, control) and 5-azacytidine tiller treatments (2.5, 5.0 and 10 μ M AC) in two winter triticale DH lines significantly differed in their responsiveness to microspore embryogenesis (ME). Electrophoregrams of recalcitrant line DH19 (a) and responsive line DH28 (b). The identified proteins were marked in the different electrophoregrams to better visualization of each protein spot. *Circles* indicate the position of identified differentially abundant proteins. Spots are described in Supplementary Table S4. **a – DH19, **b** – DH28. Figure presented full-length gels.**



b

Supplementary Table S3. The effect of tested variables on the number of differentially abundant proteins (DAPs) identified in triticale anthers isolated from tillers after 5-azacytidine treatments.

| Sources of variation | F | p |
|---------------------------------|----------|----------|
| (1) Donor plant genotype | 23.5 | *** |
| (2) Tiller treatment | 200.4 | *** |
| (1) × (2) | 2.3 | NS |

The sources of variance for the number of proteins were as follows: two DH lines, three tiller treatments, and interaction between DH line and treatment, F value calculated as variance of a variable or interaction between variables divided by error variance of the experiment, p – probability, *** $p \leq 0.001$, NS – not significant

Supplementary Table S4. List of all proteins identified in the anthers of two DH lines of winter triticale associated with stress treatment of tillers used for microspore embryogenesis (ME) induction. ME was induced by low temperature (3 weeks at 4°C) or low temperature combined with the application of 2.5, 5.0 or 10 µM of 5-azacytidine, DNA demethylating agent. Swiss-Prot database was used for protein identification. The results are based on nano LC-MS/MS analyses.

| No. | Spot no. | Protein name | Accession | MW ^a (kDa) | pI ^b | Protein Score ^c | Seq. Cov ^d [%] | Reference organism | Protein function |
|-----|----------|---|-------------|-----------------------|-----------------|----------------------------|---------------------------|-------------------------------------|-------------------------------|
| 1 | 2105 | 3-isopropylmalate dehydrogenase. chloroplastic | LEU3_SOLTU | 39.7 | 9.2 | 147.5 | 7.3 | <i>Solanum tuberosum</i> | amino acids biosynthesis |
| 2 | 7707 | Acetolactate synthase 1. chloroplastic | ILVB1_MAIZE | 68.9 | 6.8 | 57.1 | 3.4 | <i>Zea mays</i> | amino acids biosynthesis |
| 3 | 7111 | Glutamine synthetase cytosolic isozyme 1-3 | GLN13_ARATH | 38.6 | 5.7 | 100.9 | 10.2 | <i>Arabidopsis thaliana</i> | amino acids biosynthesis |
| 4 | 5311 | S-adenosylmethionine synthase 3 | METK3_HORVU | 42.7 | 5.4 | 198.7 | 7.6 | <i>Hordeum vulgare</i> | amino acids biosynthesis |
| 5 | 4507 | Betaine aldehyde dehydrogenase | BADH_HORVU | 54.3 | 5.8 | 129.9 | 5.9 | <i>Hordeum vulgare</i> | amino acids biosynthesis |
| 6 | 3701 | 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 1 | METE1_ORYSJ | 84.5 | 5.9 | 737.2 | 15.7 | <i>Oryza sativa subsp. japonica</i> | amino acids biosynthesis |
| 7 | 6506 | Ketol-acid reductoisomerase. chloroplastic | ILV5_ORYSJ | 62.3 | 6.0 | 179.7 | 5.4 | <i>Oryza sativa subsp. japonica</i> | amino acids biosynthesis |
| 8 | 6303 | Adenylosuccinate synthetase. chloroplastic | PURA_WHEAT | 50.9 | 5.9 | 44.3 | 4.0 | <i>Triticum aestivum</i> | aromatic compounds metabolism |
| 9 | 6102 | Coproporphyrinogen-III oxidase 1. chloroplastic | HEM61_ARATH | 43.8 | 6.2 | 176.9 | 5.7 | <i>Arabidopsis thaliana</i> | aromatic compounds metabolism |
| 10 | 6716 | Ribose-5-phosphate isomerase A | RPIA_RHOS5 | 27.0 | 4.7 | 45.2 | 3.8 | <i>Rhodobacter sphaeroides</i> | carbohydrate metabolism |
| 11 | 7512 | Xylose isomerase | XYLA_HORVU | 53.6 | 5.2 | 460.2 | 19.0 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 12 | 3102 | Glyceraldehyde-3-phosphate dehydrogenase 2. cytosolic | G3PC2_HORVU | 36.5 | 6.8 | 111.0 | 9.2 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 13 | 3904 | Beta-galactosidase 1 | BGAL1_ORYSJ | 91.7 | 5.7 | 140.5 | 4.4 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |

| | | | | | | | | | |
|----|------|--|-------------|------|-----|-------|------|-------------------------------------|-------------------------|
| 14 | 5904 | Putative aconitate hydratase. cytoplasmic | ACOC_ORYSJ | 98.0 | 5.6 | 89.6 | 1.1 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |
| 15 | 7402 | Enolase | ENO_ORYSJ | 47.9 | 5.3 | 361.0 | 17.7 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |
| 16 | 0206 | Glyceraldehyde-3-phosphate dehydrogenase 1. cytosolic | G3PC1_HORVU | 36.5 | 6.8 | 956.8 | 31.8 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 17 | 5507 | Phosphoglucomutase. cytoplasmic | PGMC_BROIN | 62.6 | 5.2 | 68.1 | 2.6 | <i>Bromus inermis</i> | carbohydrate metabolism |
| 18 | 7002 | Triosephosphate isomerase. cytosolic | TPIS_HORVU | 26.7 | 5.3 | 603.2 | 30.4 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 19 | 1103 | Fructose-bisphosphate aldolase cytoplasmic isozyme | ALF_ORYSJ | 36.7 | 9.0 | 239.3 | 8.9 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |
| 20 | 7706 | Transketolase. chloroplastic | TKTC_MAIZE | 72.9 | 5.4 | 72.6 | 1.9 | <i>Zea mays</i> | carbohydrate metabolism |
| 21 | 5103 | Malate dehydrogenase. cytoplasmic | MDHC_MESCR | 35.5 | 6.0 | 389.3 | 12.0 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |
| 22 | 1304 | Citrate synthase 5. mitochondrial | CISY5_ARATH | 51.7 | 6.2 | 203.8 | 6.7 | <i>Arabidopsis thaliana</i> | carbohydrate metabolism |
| 23 | 8103 | Fructose-bisphosphate aldolase. chloroplastic | ALFC_ORYSJ | 42.0 | 6.5 | 490.9 | 22.2 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |
| 24 | 9202 | Phosphoglycerate kinase, chloroplastic | PGKH_WHEAT | 49.8 | 6.7 | 577.8 | 20.0 | <i>Triticum aestivum</i> | carbohydrate metabolism |
| 25 | 4606 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1. mitochondrial | SDHA1_ARATH | 69.6 | 5.8 | 223.4 | 7.6 | <i>Arabidopsis thaliana</i> | carbohydrate metabolism |
| 26 | 6403 | Inositol-3-phosphate synthase | INO1_HORVU | 56.1 | 5.4 | 449.5 | 15.9 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 27 | 5305 | 6-phosphogluconate dehydrogenase. decarboxylating 1 | 6PGD1_ORYSJ | 52.7 | 5.8 | 229.7 | 6.5 | <i>Oryza sativa subsp. japonica</i> | carbohydrate metabolism |
| 28 | 0106 | Formate dehydrogenase. mitochondrial | FDH_HORVU | 41.5 | 7.1 | 235.0 | 11.9 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 29 | 8111 | UTP--glucose-1-phosphate uridylyltransferase | UGPA_HORVU | 51.6 | 5.1 | 965.6 | 30.2 | <i>Hordeum vulgare</i> | carbohydrate metabolism |
| 30 | 1207 | Isocitrate dehydrogenase [NAD] regulatory subunit 3. mitochondrial | IDH3_ARATH | 39.9 | 7.8 | 181.2 | 12.2 | <i>Arabidopsis thaliana</i> | carbohydrate metabolism |
| 31 | 6902 | Cell division control protein 48 homolog D | CD48D_ARATH | 90.3 | 4.9 | 186.7 | 5.2 | <i>Arabidopsis thaliana</i> | cell division |

| | | | | | | | | | |
|----|------|--|-------------|-------|-----|--------|------|-------------------------------------|------------------------|
| 32 | 8903 | Cell division control protein 48 homolog A | CD48A_ARATH | 89.3 | 5.0 | 122.3 | 5.7 | <i>Arabidopsis thaliana</i> | cell division |
| 33 | 7001 | Enoyl-[acyl-carrier-protein] reductase [NADH] 2. chloroplatic | FABI2_ORYSJ | 39.0 | 9.7 | 162.8 | 3.5 | <i>Oryza sativa subsp. japonica</i> | cytoskeleton |
| 34 | 8307 | Actin-7 | ACT7_ARATH | 41.7 | 5.2 | 442.4 | 30.2 | <i>Arabidopsis thaliana</i> | cytoskeleton |
| 35 | 5106 | Formin-like protein 1 | FH1_ARATH | 115.1 | 9.3 | 50.4 | 1.5 | <i>Arabidopsis thaliana</i> | cytoskeleton |
| 36 | 7306 | Actin-104 (Fragment) | ACT7_TOBAC | 37.1 | 5.5 | 130.9 | 4.8 | <i>Nicotiana tabacum</i> | cytoskeleton |
| 37 | 6609 | DNA repair and recombination protein RadB | RADB_THEVO | 26.0 | 8.9 | 45.9 | 2.6 | <i>Thermoplasma volcanium</i> | DNA repair |
| 38 | 2503 | ATP synthase subunit alpha. chloroplatic | ATPA_WHEAT | 55.3 | 6.1 | 279.3 | 11.7 | <i>Triticum aestivum</i> | energy processes |
| 39 | 8413 | V-type proton ATPase subunit B 1 | VATB1_HORVU | 54.0 | 5.0 | 1401.8 | 46.3 | <i>Hordeum vulgare</i> | energy processes |
| 40 | 5508 | ATP synthase subunit alpha. mitochondrial | ATPAM_MAIZE | 55.1 | 5.8 | 232.2 | 15.2 | <i>Zea mays</i> | energy processes |
| 41 | 4302 | ATP synthase subunit beta. mitochondrial | ATPBM_ORYSJ | 58.9 | 5.9 | 282.0 | 10.9 | <i>Oryza sativa subsp. japonica</i> | energy processes |
| 42 | 8609 | V-type proton ATPase catalytic subunit A | VATA_HORVU | 64.1 | 5.3 | 468.2 | 23.4 | <i>Hordeum vulgare</i> | energy processes |
| 43 | 3722 | Linoleate 9S-lipoxygenase 1 | LOX1_HORVU | 96.3 | 5.7 | 262.6 | 4.8 | <i>Hordeum vulgare</i> | fatty acids metabolism |
| 44 | 2303 | 3-oxoacyl-[acyl-carrier-protein] synthase I. chloroplatic | KASC1_HORVU | 49.0 | 7.1 | 83.6 | 4.5 | <i>Hordeum vulgare</i> | fatty acids metabolism |
| 45 | 1101 | Malate dehydrogenase. glyoxysomal | MDHG1_ARATH | 37.3 | 9.2 | 42.7 | 3.1 | <i>Arabidopsis thaliana</i> | fatty acids metabolism |
| 46 | 8202 | Ribulose bisphosphate carboxylase/oxygenase activase A. chloroplatic | RCAA_HORVU | 51.0 | 8.9 | 627.4 | 17.2 | <i>Hordeum vulgare</i> | photosynthesis |
| 47 | 2409 | Ribulose bisphosphate carboxylase large chain | RBL_WHEAT | 52.8 | 6.2 | 910.3 | 19.3 | <i>Triticum aestivum</i> | photosynthesis |
| 48 | 9006 | Oxygen-evolving enhancer protein 1. chloroplatic | PSBO_WHEAT | 34.7 | 9.5 | 791.1 | 26.8 | <i>Triticum aestivum</i> | photosynthesis |
| 49 | 8002 | Chlorophyll a-b binding protein 8. chloroplatic | CB13_SOLLC | 24.2 | 0.0 | 130.3 | 5.5 | <i>Solanum lycopersicum</i> | photosynthesis |
| 50 | 5007 | Oxygen-evolving enhancer protein 2. chloroplatic | PSBP_WHEAT | 27.3 | 9.5 | 855.9 | 44.6 | <i>Triticum aestivum</i> | photosynthesis |

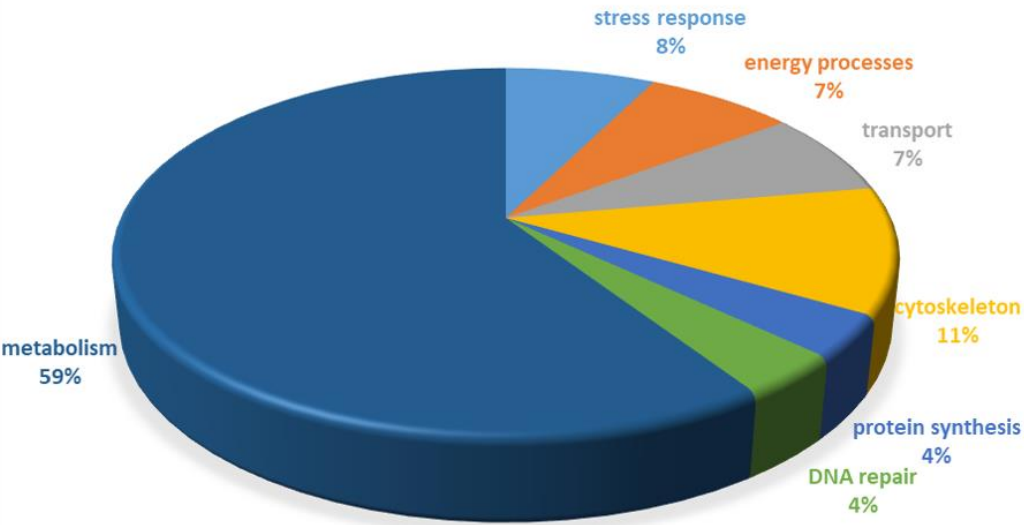
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|----|------|---|-------------|-------|------|--------|------|-------------------------------------|---------------------------|
| 51 | 2203 | 26S proteasome non-ATPase regulatory subunit 7 homolog A | PSD7A_ARATH | 34.7 | 6.0 | 201.1 | 12.7 | <i>Arabidopsis thaliana</i> | protein metabolic process |
| 52 | 0002 | Probable inactive methyltransferase | METL_ORYSJ | 40.0 | 5.6 | 60.2 | 5.1 | <i>Oryza sativa subsp. japonica</i> | protein metabolic process |
| 53 | 3204 | Protein disulfide-isomerase like 2-1 | PDI21_ARATH | 39.5 | 5.7 | 63.5 | 2.8 | <i>Arabidopsis thaliana</i> | protein metabolic process |
| 54 | 1002 | Putative F-box/FBD/LRR-repeat protein At4g26350 | FDL28_ARATH | 49.6 | 9.7 | 40.4 | 2.1 | <i>Arabidopsis thaliana</i> | protein metabolic process |
| 55 | 3305 | 26S protease regulatory subunit 7 | PRS7_ORYSJ | 47.7 | 5.9 | 194.2 | 11.0 | <i>Oryza sativa subsp. japonica</i> | protein metabolic process |
| 56 | 9504 | RuBisCO large subunit-binding protein subunit beta. chloroplastic | RUBB_SECCE | 53.4 | 4.7 | 530.4 | 19.2 | <i>Secale cereale</i> | protein metabolic process |
| 57 | 7705 | Chaperone protein ClpC1. chloroplastic | CLPC1_ORYSJ | 101.7 | 6.1 | 452.7 | 12.0 | <i>Oryza sativa subsp. japonica</i> | protein metabolic process |
| 58 | 7611 | 70 kDa peptidyl-prolyl isomerase | FKB70_WHEAT | 62.0 | 5.2 | 460.9 | 21.6 | <i>Triticum aestivum</i> | protein synthesis |
| 59 | 7308 | Elongation factor Tu. chloroplastic | EFTU_PEA | 53.0 | 6.7 | 166.4 | 5.7 | <i>Pisum sativum</i> | protein synthesis |
| 60 | 4111 | Elongation factor Tu. mitochondrial | EFTM_ARATH | 49.4 | 6.3 | 385.0 | 9.7 | <i>Arabidopsis thaliana</i> | protein synthesis |
| 61 | 3502 | Oleosin GRP-17 | GRP17_ARATH | 53.2 | 10.9 | 90.0 | 5.3 | <i>Arabidopsis thaliana</i> | reproduction sexual |
| 62 | 9613 | Heat shock 70 kDa protein 2 | HSP72_USTMA | 70.3 | 4.9 | 282.7 | 9.5 | <i>Ustilago maydis</i> | stress response |
| 63 | 3601 | Hsp70-Hsp90 organizing protein 1 | HSOP1_SOYBN | 64.1 | 5.4 | 74.5 | 2.6 | <i>Glycine max</i> | stress response |
| 64 | 7604 | Heat shock 70 kDa protein 10. mitochondrial | HSP7J_ARATH | 72.9 | 5.5 | 446.1 | 6.2 | <i>Arabidopsis thaliana</i> | stress response |
| 65 | 7004 | Lactoylglutathione lyase | LGUL_ORYSJ | 32.5 | 5.4 | 326.8 | 11.3 | <i>Oryza sativa subsp. Japonica</i> | stress response |
| 66 | 9611 | Heat shock 70 kDa protein 3 | HSP7C_ARATH | 71.1 | 4.8 | 1169.7 | 25.4 | <i>Arabidopsis thaliana</i> | stress response |
| 67 | 3306 | Cytosolic isocitrate dehydrogenase [NADP] | ICDHC_ARATH | 45.7 | 6.1 | 548.5 | 19.0 | <i>Arabidopsis thaliana</i> | stress response |
| 68 | 601 | Protein TOC75. chloroplastic | TOC75_ORYSJ | 87.6 | 9.2 | 211.8 | 6.7 | <i>Oryza sativa subsp. japonica</i> | transport |
| 69 | 5401 | Guanosine nucleotide diphosphate dissociation inhibitor At5g09550 | GDI_ARATH | 49.5 | 5.0 | 72.6 | 8.3 | <i>Arabidopsis thaliana</i> | transport |

^aThe theoretical molecular weight (MW. kDa) and ^b isoelectric point (pI) retrieved from the protein database

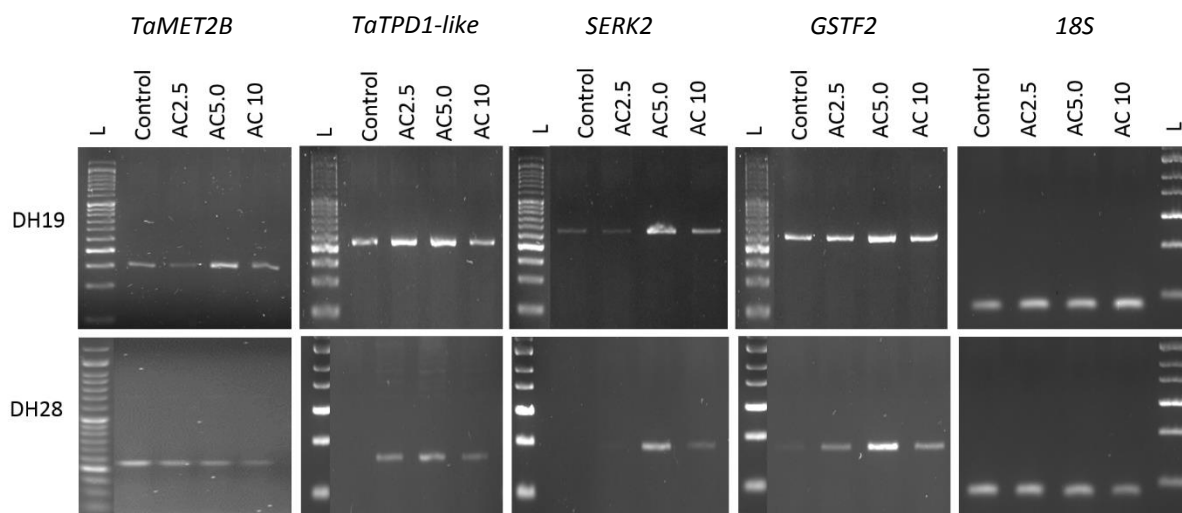
^cThe score and ^dprotein sequence coverage (Score in Flex Analysis software)

The protein name indicated with **bold** – the proteins previously identified as associated with ME¹⁶.

Supplementary Figure S2. Diagrams showing functional classification of identified differentially abundant proteins in both studied triticale DH lines after AC treatments.



Supplementary Figure S3. The effect of low temperature (3 weeks at 4 °C, Control) and 5-azacytidine (AC) tiller treatments on the expression level of selected genes (*TaMET2B*, *TaTPD1-like*, *SERK2*, *GSTF2*) associated with microspore embryogenesis (ME) induction in the anthers of two winter triticale doubled haploid (DH) lines (responsive DH28 and recalcitrant DH19). AC was applied at concentrations of 2.5 μM (AC2.5), 5.0 μM (AC5.0) and 10 μM (AC10). *18S rDNA* was used as a control. Figure presented full-length gels.



Supplementary Table S5. Primers used in this study.

| Primer name | Sequence 5' to 3' | References |
|----------------------|--------------------------|-----------------------------|
| <i>TaMET2B_F</i> | TGTGCCACCCCCTCTTGC | Thomas et al. ⁶⁵ |
| <i>TaMET2B_R</i> | CAGTTCATGCCAGCCATGC | Thomas et al. ⁶⁵ |
| <i>TaTPD1-like_F</i> | AAGTTTCAGCGTCTTCCTCGCT | Žur et al. ¹² |
| <i>TaTPD1-like_R</i> | TCGATGCAGGTGTTGGTGA ACT | Žur et al. ¹² |
| <i>SERK2_F</i> | AGCTTCGATTCCTCCGTCTT | Žur et al. ¹² |
| <i>SERK2_R</i> | AGGCACCTGCTGATTGAGTT | Žur et al. ¹² |
| <i>GSTF2_F</i> | CGGCAAAGCTGACGAATCTGTT | Žur et al. ¹² |
| <i>GSTF2_R</i> | ACTTCTCTGCCTTCTTTCCGAACC | Žur et al. ¹² |
| <i>18SrDNA_F</i> | CGGCTACCACATCCAAGGAA | Žur et al. ¹² |
| <i>18SrDNA_R</i> | TGTCACTACCTCCCCGTGTCA | Žur et al. ¹² |