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3 1 **An up-to-date workflow for plant (phospho)proteomics identifies differential drought-**
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5 2 **responsive phosphorylation events in maize leaves**
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26 **ABSTRACT**

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28 Protein phosphorylation is one of the most common post-translational modifications (PTMs),
29 which can regulate protein activity and localization, as well as protein–protein interactions in
30 numerous cellular processes. Phosphopeptide enrichment techniques enabled plant researchers
31 to acquire insight in phosphorylation-controlled signaling networks in various plant species.
32 Most phosphoproteome analyses of plant samples still involve stable isotope labeling, peptide
33 fractionation, and demand lots of mass spectrometry (MS) time. Here, we present a simple
34 workflow to probe, map and catalogue plant phosphoproteomes, requiring relatively low
35 amounts of starting material, no labeling, no fractionation, and no excessive analysis time.
36 Following optimization of the different experimental steps on *Arabidopsis thaliana* samples,
37 we transferred our workflow to maize, a major monocot crop, to study signaling upon drought
38 stress. In addition, we included normalization to protein abundance to identify true
39 phosphorylation changes. Overall, we identified a set of new phosphosites in both
40 *Arabidopsis thaliana* and maize, some of which are differentially phosphorylated upon
41 drought. All data are available via ProteomeXchange with identifier PXD003634, but to
42 provide easy access of the whole scientific community to our model plant and crop datasets,
43 we created an online database, Plant PTM Viewer
44 (bioinformatics.psb.ugent.be/webtools/ptm_viewer/), where all phosphosites identified in our
45 study can be consulted.

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47 **Key Words:** Phosphoproteomics, maize, Arabidopsis, drought stress, database

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3 51 **INTRODUCTION**
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7 53 The balanced action of protein kinases and phosphatases determines a proteome's
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10 54 phosphorylation status. Protein phosphorylation may transiently modify protein properties,
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12 55 such as enzymatic activity, subcellular localization, protein structure and stability, and
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14 56 interactions with other proteins. As such, many cellular signaling processes, such as
15
16 57 transmembrane signaling, intracellular amplification of signals and cell cycle control, occur
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18 58 via reversible protein phosphorylation (1). In plants, phosphorylation-mediated signaling is of
19
20 59 central importance in various physiological processes, including hormone signaling and stress
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22 60 responses (2). However, only a limited number of kinases and phosphatases (and their targets)
23
24 61 have been studied in different levels of detail (3).
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27 62 Mass spectrometry (MS)-based proteomics became an essential tool for studying
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29 63 protein phosphorylation and has enabled the identification of numerous phosphorylation sites
30
31 64 on plant proteins (3). Nevertheless, studies of phosphorylation events remain challenging, due
32
33 65 to their dynamic nature and the sub-stoichiometric levels of phosphorylated proteins.
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35 66 Therefore, at least some level of enrichment for phosphorylation sites is needed and this is
36
37 67 best done at the peptide level to maximize the identification of phosphosites. The most
38
39 68 productive approach is based on metal (ion) chelation. By exploiting the interaction between
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41 69 negatively charged phosphate groups and positively charged metal ions or metal oxides,
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43 70 immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography
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45 71 (MOAC) methods, respectively, represent efficient ways to enrich phosphopeptides from
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47 72 complex mixtures. Enrichment with TiO₂ beads became a routine method in plant proteomics
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49 73 studies in recent years (4-12). In an attempt to maximally cover phosphoproteomes, the
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51 74 majority of phosphoproteomics approaches make use of peptide fractionation methods, such
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53 75 as strong cation exchange chromatography, hydrophilic interaction chromatography or
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3 76 reversed-phase chromatography (7, 13-17). These however result in far more LC-MS/MS
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5 77 measurement time per sample to be analyzed and also require large(r) amounts of starting
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7 78 material.

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10 79 With the ever-increasing number of phosphorylation sites being identified – for nearly
11
12 80 every human cellular protein a phosphosite has been reported (18) – the functionality of these
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14 81 post-translational modifications (PTMs) is questioned. Crowdedness effects are hypothesized
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16 82 to give rise to non-functional transfer of a phosphate group by kinases upon encounter of a
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18 83 random protein (19, 20). Merely profiling phosphorylation sites will hence likely lead to the
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20 84 large scale identification of nonfunctional PTMs. To discriminate these ‘noisy’ phosphosites
21
22 85 from sites with regulatory significance, experiments where differential conditions are
23
24 86 compared are vital. Obviously, this requires assessing dynamics in the phosphoproteome via
25
26 87 quantitative methods. Methodologies for the quantitative analysis of phosphoproteomes in
27
28 88 plants are most frequently based on stable isotope labeling, like $^{15}\text{N}/^{14}\text{N}$ metabolic labeling of
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30 89 proteins during plant growth (4, 11, 16, 21, 22) or post-metabolic labeling of peptides with
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32 90 iTRAQ (9, 12, 23, 24). As labeling imposes limitations on the number of conditions that can
33
34 91 be monitored, label-free methods represent a practical alternative. Two label-free methods,
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36 92 spectral counting and precursor ion intensity-based quantification, have been applied in plant
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38 93 phosphoproteome strategies (8, 10, 11, 14, 25-27). However, label-free approaches often
39
40 94 suffer from quantitative incompleteness due to stochastic data acquisition (MS/MS
41
42 95 sequencing) leading to numerous missing values in the dataset, which – to some extent – can
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44 96 be avoided by matching data between LC-MS(/MS) runs.

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49 97 Although missing in most published plant phosphoproteome studies [some exceptions
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51 98 are (12, 15, 16)], parallel and in depth investigation of the overall proteome is recommended
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53 99 for normalization of quantitative PTM studies. To determine if phosphopeptide changes are
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55 100 the result of true phosphorylation changes or rather general abundance changes of the
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3 101 phosphoprotein, phosphopeptide levels need to be normalized to overall protein abundances.
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5 102 Ideally, such changes in overall protein levels should be derived from an analysis of non-
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7 103 phosphopeptides of the same sample (28).
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9
10 104 Agricultural plants, such as maize, routinely face drought stress, which is one of the
11
12 105 worst environmental hazards that impacts crop productivity (29, 30). Some crop cultivars are
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14 106 known to better withstand abiotic stress, but these responses are dynamic and complex and
15
16 107 often a genetic basis is hard to find (31). As plants remodel their proteome in response to
17
18 108 stress, drought-adaptive traits are likely to be reflected at the proteome level (32). Moreover,
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20 109 as a universal biochemical signal in cells, protein phosphorylation controls stress responses,
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22 110 transmitting stress signals from the cell surface to the nucleus (33).
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24
25 111 Taken together, commonly used strategies in plant phosphoproteomics involve tedious
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27 112 labeling approaches and fractionation steps, which are time consuming, expertise demanding
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29 113 and negatively affect reproducibility, robustness and throughput. Here, we present a label-free
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31 114 quantitative workflow for quick and reproducible phosphoproteome analysis of plant tissue,
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33 115 requiring only small sample amounts and no costly expert software for data analysis and
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35 116 integrating steps (such as normalization) that are not yet standard in plant phosphoproteomics.
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38 117 We applied our workflow to maize, a major monocot crop, to study signaling upon drought
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40 118 stress, and we identified a set of new phosphosites in maize, some of which are differentially
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42 119 phosphorylated upon drought.
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47 121 **EXPERIMENTAL SECTION**

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52 123 **Plant growth**

54 124 Seedlings of *A. thaliana* (ecotype Columbia) were grown on vertically-held plates with half-
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56 125 strength Murashige and Skoog medium solidified with 0.8% agar at 22°C in continuous light.
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3 126 Four days post germination (dpg), the plants were transferred to 10 μ M 1-naphthaleneacetic
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5 127 acid (NAA)-containing plates. Roots were harvested 5 dpg. Maize plants (inbred line B104)
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7 128 were grown in soil in a growth chamber with controlled relative humidity (55%) and
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9 129 temperature (24°C), in a 16h/8h (day/night) cycle. Drought was induced by lowering the soil
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11 130 water capacity to 62.5% relative to that of the well-watered control plants. 21 days after
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13 131 sowing, the first 4 cm of growing leaf 7 was harvested.
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18 133 **Protein Extraction and Tryptic Digestion**

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20 134 Plant material was harvested in three biological replicates. One g of fresh weight material was
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22 135 flash-frozen in liquid nitrogen, and manually ground into a fine powder with a pestle and
23
24 136 mortar. Proteins were extracted in homogenization buffer containing 50 mM Tris-HCl buffer
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26 137 (pH 8), 0.1 M KCl, 30% sucrose, 5 mM EDTA, and 1 mM DTT in milliQ water, and the
27
28 138 appropriate amounts of the Complete protease inhibitor mixture and the PhosSTOP
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30 139 phosphatase inhibitor mixture (both from Roche) were added. The samples were sonicated on
31
32 140 ice and centrifuged at 4°C for 15 min at 2,500 \times g to remove debris. Supernatants were
33
34 141 collected and a methanol/chloroform precipitation was carried out by adding 3, 1 and 4
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36 142 volumes of methanol, chloroform and water, respectively. Samples were centrifuged for
37
38 143 10 min at 5,000 \times g, and the aqueous phase was removed. After addition of 4 volumes
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40 144 methanol, the proteins were pelleted via centrifugation for 10 min at 2,500 \times g. Pellets were
41
42 145 washed with 80% acetone and re-suspended in 6 M guanidinium hydrochloride in 50 mM
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44 146 triethylammonium bicarbonate (TEAB) buffer (pH 8). Alkylation of cysteines was carried out
45
46 147 by adding a combination of tris(carboxyethyl)phosphine (TCEP, Pierce) and iodoacetamide
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48 148 (Sigma-Aldrich) to final concentrations of 15 mM and 30 mM respectively, and the reaction
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50 149 was allowed for 15 min at 30°C in the dark. Before digestion, the samples were buffer
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52 150 exchanged on Illustra NAP columns (GE Healthcare Life Sciences) to 50 mM TEAB buffer
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3 151 (pH 8) and the protein concentration was measured using the Bio-Rad Protein Assay. One mg
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5 152 of the proteins was pre-digested with EndoLysC (Wako Chemicals) for 4 h, followed by a
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7 153 digestion with trypsin overnight (Promega Trypsin Gold, mass spectrometry grade), both
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9 154 digestions occurring at 37°C at an enzyme-to-substrate ratio of 1:100 (w:w). The digest was
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11 155 acidified to pH \leq 3 with trifluoroacetic acid (TFA) and desalted with SampliQ C18 SPE
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13 156 cartridges (Agilent) according to the manufacturer's guidelines. The eluates were split into
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15 157 two and dried in a vacuum centrifuge. One half of the samples served for proteome analyses
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17 158 and were re-dissolved in 30 μ L of 2% (v/v) acetonitrile and 0.1% (v/v) TFA right before LC-
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19 159 MS/MS analysis.
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24 25 161 **Phosphopeptide Enrichment**

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27 162 The dried eluates were resuspended in 100 μ L of loading solvent (80% acetonitrile, 5% TFA)
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29 163 and incubated with 1 mg MagReSyn® Ti-IMAC microspheres for 20 min at room
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31 164 temperature. The microspheres were next washed once with wash solvent 1 (80% acetonitrile,
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33 165 1% TFA, 200 mM NaCl) and two times with wash solvent 2 (80% acetonitrile, 1% TFA). The
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35 166 bound phosphopeptides were eluted with three volumes (80 μ L) of a 1% NH₄OH solution,
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37 167 followed immediately by acidification to pH \leq 3 with formic acid. Prior to MS analysis, the
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39 168 samples were vacuum-dried and re-dissolved in 50 μ L of 2% (v/v) acetonitrile and 0.1% (v/v)
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41 169 TFA.
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46 47 171 **Mass Spectrometry**

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49 172 Each sample was analyzed twice (i.e. in two technical replicates) via LC-MS/MS on an
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51 173 Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific) in-line connected to a Q Exactive
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53 174 mass spectrometer (Thermo Fisher Scientific). The sample mixture was first loaded on a
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55 175 trapping column (made in-house, 100 μ m internal diameter (I.D.) \times 20 mm, 5 μ m beads C18
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3 176 Reprisil-HD, Dr. Maisch, Ammerbuch-Entringen, Germany). After flushing from the
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5 177 trapping column, the sample was loaded on an analytical column (made in-house, 75 μ m I.D.
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7 178 \times 150 mm, 3 μ m beads C18 Reprisil-HD, Dr. Maisch). Peptides were loaded with loading
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9 179 solvent A (0.1% TFA in water) and separated with a linear gradient from 98% solvent A'
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11 180 (0.1% formic acid in water) to 55% solvent B' (0.1% formic acid in water/acetonitrile, 20/80
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13 181 (v/v)) in 170 min at a flow rate of 300 nL/min. This was followed by a 5 min wash reaching
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15 182 99% solvent B'. The mass spectrometer was operated in data-dependent, positive ionization
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17 183 mode, automatically switching between MS and MS/MS acquisition for the 10 most abundant
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19 184 peaks in a given MS spectrum. The source voltage was 3.4 kV, and the capillary temperature
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21 185 was 275°C. One MS1 scan (m/z 400–2000, AGC target 3×10^6 ions, maximum ion injection
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23 186 time 80 ms) acquired at a resolution of 70000 (at 200 m/z) was followed by up to 10 tandem
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25 187 MS scans (resolution 17500 at 200 m/z) of the most intense ions fulfilling predefined
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27 188 selection criteria (AGC target 5×10^4 ions, maximum ion injection time 60 ms, isolation
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29 189 window 2 Da, fixed first mass 140 m/z, spectrum data type: centroid, underfill ratio 2%,
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31 190 intensity threshold 1.7×10^4 , exclusion of unassigned, 1, 5-8, >8 charged precursors, peptide
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33 191 match preferred, exclude isotopes on, dynamic exclusion time 20 s). The HCD collision
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35 192 energy was set to 25% Normalized Collision Energy and the polydimethylcyclosiloxane
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37 193 background ion at 445.120025 Da was used for internal calibration (lock mass).
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195 **Data Analysis**

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47 196 For the Arabidopsis samples, MS/MS spectra were searched against a Uniprot database
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49 197 containing *A. thaliana* sequences (34,509 entries, version November, 2014) with the
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51 198 MaxQuant software (version 1.5.3.8). For the maize samples, the searches were done against
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53 199 a *Zea mays* database downloaded from PLAZA Monocots 3.0 (34) containing sequences
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55 200 (39,305 entries, version 2014) with the MaxQuant software (version 1.5.0.30). For all
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3 201 searches, a precursor mass tolerance was set to 20 ppm for the first search (used for nonlinear
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5 202 mass re-calibration) and set to 4.5 ppm for the main search. Trypsin was selected as enzyme
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7 203 setting. Cleavages between lysine/arginine-proline residues and up to two missed cleavages
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9 204 were allowed. Carbamidomethylation of cysteine residues was selected as a fixed
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11 205 modification and oxidation of methionine residues was selected as variable modification. For
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13 206 the samples enriched for phosphopeptides phosphorylation of serine, threonine and tyrosine
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15 207 residues were set as additional variable modifications. The false discovery rate for peptide and
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17 208 protein identifications was set to 1%, and the minimum peptide length was set to 7. The
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19 209 minimum score threshold for both modified and unmodified peptides was set to 30. The
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21 210 MaxLFQ algorithm allowing label-free quantification (35) and the ‘Matching Between Runs’
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23 211 feature were enabled. All mass spectrometry proteomics data have been deposited to the
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25 212 ProteomeXchange Consortium via the PRIDE (36) partner repository with the dataset
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27 213 identifier PXD003634. For the quantitative maize proteome and phosphoproteome analyses,
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29 214 the ‘ProteinGroups’ and ‘Phospho(STY)sites’ output files, respectively, generated by the
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31 215 MaxQuant search was loaded into Perseus, the data analysis software available in the
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33 216 MaxQuant package. Only proteins or phosphosites which were quantified in at least two of
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35 217 the three biological replicates of at least one sample were retained. Log₂ transformed protein
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37 218 LFQ intensities or phosphosites intensities were centered by subtracting the median of the
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39 219 entire set of protein/phosphosite intensities per sample. A two-sample test with $p < 0.05$ was
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41 220 carried out to test the differences between groups. The statistically significant hits were then
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43 221 Z-scored and clustered into groups by a hierarchical clustering analysis based on Euclidean
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45 222 distance.

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52 223 To identify novel phosphosites (not previously reported ones), we compared our data
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54 224 to the PhosPhAt 4.0 full dataset of experimentally identified phosphosites (37) (data from
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3 225 04.04.2016) and to the retrieved database of phosphosites identified in maize seed and leaf
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5 226 tissues from the Maize Protein Atlas (17, 38) (data from 04.04.2016).
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9 228 **Normalization of phosphoproteome data**

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11 229 We accounted for protein expression changes to allow proper interpretation of the maize
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13 230 quantitative phosphoproteomics data. After log₂ transformation and centralization, the
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15 231 intensities of the phosphosites were normalized by subtracting the log₂ transformed and
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17 232 centralized LFQ intensities of the corresponding proteins. The latter dataset of protein LFQ
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19 233 intensities resulted from the parallel protein expression study of all maize samples.
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23 235 **Gene Ontology Analysis**

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27 236 GO enrichment analysis was performed in the PLAZA 3.0 workbench (34). For the proteome
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29 237 dataset, 234 proteins with significant changes in abundance were analyzed, using the dataset
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31 238 of 2299 identified proteins as background model. A FDR cutoff ≤ 0.04 was used to score
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33 239 significantly overrepresented or depleted GO terms. For the phosphoproteome dataset, the
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35 240 identified proteins in each conditions were used for the enrichment, and the whole theoretical
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37 241 proteome (based on the genome annotation of *Z. mays*) was used at background. P-value
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39 242 cutoff was set at 0.04 and only terms enriched in either condition were presented (Table
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41 243 Sxxx).
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46 245 **Motif-X analysis**

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49 246 The Motif-X algorithm (39) was used to extract significantly enriched amino acid motifs
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51 247 surrounding the identified phosphosites. The sequence window was limited to 13 amino acids
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53 248 and foreground peptides were pre-aligned with the phosphosite centered. *Zea mays* proteome
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3 249 data set from PLAZA was used as the background database. The occurrence threshold was set
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5 250 at the minimum of 20 peptides and the P-value threshold was set at $< 10^{-6}$.

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8 9 252 **STRING analysis of protein-protein interaction networks**

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11 253 Protein-protein interactions were analyzed by STRING (<http://string-db.org/>) (40), using the
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13 254 sequences of differentially phosphorylated proteins and proteins with significant abundance
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15 255 changes as input. The required confidence score was set as > 0.700 for highly confident
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17 256 interactions (STRING protein-protein interaction prediction is based on data available for
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19 257 genomic homology, gene fusion, occurrence in the same metabolic pathways, co-expression,
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21 258 experiments, database and text mining. A combined score is calculated based on the score of
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23 259 all the methods that were used for the protein-protein interaction prediction. The higher the
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25 260 score is, the more confident the interaction). The results were visualized using the Cytoscape
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27 261 package.
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33 34 263 **Pubmed search**

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36 264 A Pubmed search (www.ncbi.nlm.nih.gov/pubmed/) was performed on 31/03/2016 using
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38 265 ‘maize proteomics 2015’ or ‘proteomics arabidopsis 2016’ to identify relevant papers (only
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40 266 research papers with the correct focus were retained).
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44 45 268 **RESULTS AND DISCUSSION**

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48 49 270 *Optimized quantitative workflow for proteomics and phosphoproteomics in plants*

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54 272 To facilitate efficient proteome analyses of plants, we developed a simple workflow, which
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56 273 maximizes the coverage and reproducibility of protein and phosphorylation site quantification
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3 274 in single LC-MS/MS runs, thus without requiring peptide fractionation steps (**Figure 1**). To
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5 275 optimize the pipeline, we used the fully sequenced and well-annotated model plant
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7 276 *Arabidopsis thaliana* (see next section for results).
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10 277 First, we provide a brief overview of the key steps in the protocol and the
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12 278 improvements that were introduced step-by-step to robustly survey plant proteomes and
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14 279 phosphoproteomes (more details can be found in the **Experimental Section**). To reproducibly
15
16 280 capture a comprehensive spectrum of proteins, we opted for a protein precipitation approach.
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18 281 Plant tissue is known to be more challenging for proteome analyses than yeast and
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20 282 mammalian cells, with plant cells holding low protein contents and high concentrations of
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22 283 compounds that hinder preparation of proteome samples (e.g. polysaccharides, phenolic
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24 284 compounds, lipids and secondary metabolites) (41). From plant material that was grinded into
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26 285 powder, proteins were extracted with a sucrose buffer, containing protease and phosphatase
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28 286 inhibitors. The extract was subsequently purified through a chloroform/methanol precipitation
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30 287 step and the pelleted proteins were reconstituted in a buffer containing guanidinium
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32 288 hydrochloride. Cysteine disulfide bonds were reduced with tris(2-carboxyethyl)phosphine
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34 289 hydrochloride (TCEP-HCl), allowing the alkylation reaction with iodoacetamide to
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36 290 simultaneously take place (42). Next, we pre-digested the proteins with endoproteinase-LysC
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38 291 for 4 hours, followed by a full digestion with trypsin for 14 hours. The pre-digestion step was
39
40 292 previously shown to substantially improve the proteolytic efficiency of trypsin (43). The
41
42 293 resulting peptides were desalted, and split into two. One part was used for the proteome
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44 294 analysis, leaving 500 μg of digest material as input for phosphopeptide enrichment. We opted
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46 295 for a Ti^{4+} -IMAC-based method, as it was found to perform extremely well in terms of
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48 296 reproducibility and provides even greater selectivity and sensitivity than the more commonly
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50 297 used TiO_2 chromatography (44, 45). Both the proteome and phosphoproteome samples were
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52 298 analyzed by 3 hour gradients on a quadrupole Orbitrap instrument [Q Exactive (46)].
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3 299 Second, peptide identification and quantification are important steps following LC-
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5 300 MS/MS analysis. We chose a label-free quantitation approach over a labeling method, as it is
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7 301 cost-effective, does not restrict the numbers of samples that can be compared, and can span
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9 302 several orders of magnitude of protein concentrations (35). In most label-free studies of plant
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11 303 phosphoproteomes, the raw data are analyzed by a combination of expensive expert peptide
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13 304 identification software, like Proteome Discoverer or Mascot, and in-house developed
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15 305 algorithms to facilitate label free quantification (10, 11, 26, 27) (**Supplementary Table S1**).
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17 306 Here, peptide identification was carried out by the freely available and easy-to-use software
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19 307 package MaxQuant (47). Simultaneously, the label-free quantitation is carried out by
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21 308 MaxQuant, in an ion intensity-based manner (35). The missing value issue, due to stochastic
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23 309 peptide sequencing inherent to mass spectrometry, was tackled by using the “match between
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25 310 runs” feature in MaxQuant, which can transfer MS/MS identifications between measurements
26
27 311 based on a peptide retention time correlation approach (35).
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32 312 Taken together, compared to published methods for (phospho)proteomics in plants
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34 313 (12, 14-16, 23), we reduced the number of sample preparation steps, MS time and data
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36 314 analysis complexity, due to the lack of labeling, gel-based steps and pre-fractionation steps
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38 315 and the introduction of MaxQuant in our workflow. With respect to the latter, this facilitates
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40 316 and standardizes data analysis, but does (not yet) seem to be routinely integrated in plant
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42 317 proteomics (**Supplementary Table S1**).
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47 319 *Validating the optimized workflow on Arabidopsis thaliana roots*

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52 321 To validate our complete (phospho)proteomics pipeline we used 100 mg *Arabidopsis* roots of
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54 322 five-day old seedlings, which were harvested in three independent biological replicates and
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56 323 yielded 1 mg proteins per sample. All samples were analyzed twice via nano-LC-MS/MS
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3 324 using three hour gradients. We first analyzed the non-enriched samples, amounting to a total
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5 325 of 18 hours of MS time and leading to the cumulative identification of 34,216 unique peptides
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7 326 (with an estimated false discovery rate of 1%) that could be mapped on 4,903 protein groups
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9 327 (**Supplementary Table S2**). The latter can be defined as protein entries distinguishable on the
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11 328 basis of identified peptides (48). Via the MaxLFQ algorithm, 4,847 of those could be
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13 329 quantified in at least one biological replicate and 2,992 in all replicates. Seeing that label-free
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15 330 methods are very replicate dependent, reproducibility of the chromatographic separation must
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17 331 be very high. The data from the replicate experiments clearly show highly accurate
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19 332 quantitative reproducibility with an average Pearson correlation of 0.978 (**Supplementary**
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21 333 **Figure S1**).

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25 334 A common challenge for plant proteomics studies is the difficulty of isolating proteins
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27 335 from the different subcellular organelles with sufficient efficiency. Membrane proteins
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29 336 represent an additional hurdle, as their large size and hydrophobicity render them difficult to
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31 337 isolate. To obtain a wide-ranging snapshot of cellular signaling processes it is vital to capture
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33 338 proteins from not only the cytosol, but also from membranes and organelles. GO analysis
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35 339 shows that the applied protocol extracted proteins from cytoplasm, nucleus, plasma membrane
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37 340 and other organelles (**Supplementary Table S3**). This evidences that our approach is not
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39 341 limited by particular experimental difficulties and recovers proteins from all subcellular
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41 342 membranes and organelles.

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45 343 Next, we monitored the phosphorylation events in the *Arabidopsis* samples. The total
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47 344 of six LC-MS/MS runs of the Ti^{4+} -IMAC enriched samples resulted in the identification of
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49 345 1,051 unique phosphopeptides, corresponding to 1,331 phosphosites on 706 protein groups, at
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51 346 an estimated false discovery rate of 1%, for both peptide-spectrum match and protein
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53 347 (**Supplementary Table S4**). The vast majority of these sites occurred on serine and threonine
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55 348 residues (90.3% and 9.1%, respectively), whereas phosphotyrosines accounted for less than

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3 349 1% of the identified sites. This is in agreement with other reports (9, 13). Accurate site
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5 350 localization (probability > 0.75) was achieved for 799 of these phosphosites on 552 proteins.
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7 351 Of the 1,331 unique phosphosites, we could accurately quantify 1,022 and 711 in at least one
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9 352 and in all biological replicates, respectively. To evaluate the quality of the experiment, we
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11 353 assessed the correlation of all phosphopeptide intensities between the three biological
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13 354 replicates. An average Pearson's correlation of 0.818 illustrates the high reproducibility of the
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15 355 phosphopeptide enrichment strategy (**Supplementary Figure S1**). All the identified
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17 356 *Arabidopsis* phosphosites were used to search against the PhosPhAt 4.0 full dataset of
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19 357 experimentally identified phosphosites (37). This resulted in 169 phosphosites (13% of the
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21 358 dataset) uniquely identified in our study (**Supplementary Table S5**).
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25 359 In summary, we have experimental evidence that our workflow successfully detects a
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27 360 large portion of the (phospho)proteome and can thus be applied to understanding biological
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29 361 processes.
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34 363 *Applying the (phospho)proteomics workflow to maize leaves under drought stress*

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38 365 Following the validation of our pipeline in *Arabidopsis* roots, we applied our workflow to a
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40 366 monocot crop under stress. Given the importance of drought-related research (33, 49-51), we
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42 367 profiled the proteome and phosphoproteome of maize leaves subjected to (severe) drought
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44 368 stress. Since the growth zone of the maize leaf determines to a great extent the final leaf
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46 369 length (52) and drought affects cell division and cell expansion in the growth zone of the
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48 370 maize leaf (53), we harvested the growth zone (4 basal centimeters) of the growing leaf 7 of
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50 371 21 day old plants grown under drought conditions and control plants (three independent
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52 372 biological replicates were collected for both conditions). The drought was applied by
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54 373 preventing irrigation upon sowing and when the soil water content reached 62.5% of that of
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3 374 the well-watered controls, the plants were maintained at the respective watering regime by
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5 375 daily watering. At the moment leaf 7 appeared, the effects of the drought were quantified by
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7 376 measuring the final leaf length of the youngest fully grown leaf, leaf 4. This leaf showed a
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9 377 significant length reduction compared to the control (**data not shown**), supporting that the
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11 378 applied drought affected leaf growth.

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14 379 Proteome and phosphoproteome data were obtained for growth zones of maize leaves
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16 380 as described above, further emphasizing the importance of moving away from gel-based
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18 381 approaches, also in crops where this is not standard yet (**Supplementary Table S1**). All
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20 382 biological samples were analyzed twice by nanoLC-MS/MS using three hour gradients.
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24 384 *The maize leaf proteome under drought stress*

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29 386 In the non-enriched samples, a total of 22,093 peptides were identified originating from 4,409
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31 387 protein groups. 4,361 protein groups could be accurately quantified, of which 2,299 in at least
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33 388 two of the three biological replicates (**Supplementary Table S6**). The data from the replicate
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35 389 experiments show quantitative reproducibility with an average Pearson correlation of 0.856
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37 390 and 0.892 for control and drought samples, respectively (**Supplementary Figure S2**).
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39 391 Statistical testing ($p < 0.05$) pinpointed 234 of these proteins to be differently regulated, with
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41 392 156 up- and 78 down-regulated proteins upon drought stress (**Figure 2A and Supplementary**
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43 393 **Table S7-S8**). GO analysis of these proteins showed an overrepresentation of proteins
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45 394 involved in carbohydrate metabolism and chromatin remodeling (**Supplementary Table S9**),
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47 395 in agreement with previous studies of drought responses in crops (54-56). Furthermore,
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49 396 amongst the proteins with increased abundance we observed proteins associated with water
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51 397 deprivation, like lipoxygenase (GRMZM2G015419; (57)), keto reductase family 4 member
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53 398 C9 (GRMZM2G059314; (58)), fructose-biphosphate aldolase (GRMZM2G057823; (59)) and
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3 399 protein-L-isoaspartate methyltransferase (GRMZM2G423027; (60)). Interestingly, BRI1-
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5 400 associated receptor kinase (BAK1) (GRMZM2G089819), known for its role in plant defense
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7 401 responses and brassinosteroid signaling (61, 62), was found to be down regulated in water
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9 402 deprived conditions. The brassinosteroid receptors, BRIs, to which BAK1 binds upon
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11 403 brassinosteroid induction, play an important role in maize leaf growth (63). Moreover,
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13 404 brassinosteroid signaling has previously been linked to abiotic stress (64-66) and
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15 405 brassinosteroid application is reported to improve drought tolerance in wheat and the
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17 406 resurrection grass *Sporobolus stapfianus* (67).
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21 407 Analysis of protein-protein interactions between the 234 proteins with significant
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23 408 abundance changes resulted in a network of 141 proteins and 277 interactions, whereas each
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25 409 interaction has a combined score of all prediction methods > 0.7 (see **Experimental Section**)
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27 410 (**Figure 3**). The network is approximately centralized around the DNA topoisomerase II
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29 411 (GRMZM2G021270/PLAZA identifier ZM05G37510). From the resulted network, we
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31 412 identified different groups of interaction between proteins involved in different cellular
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33 413 processes. These included the categories DNA/chromatin organization, photosynthesis and
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35 414 glucose metabolism, of which the corresponding GO terms were enriched in the dataset.
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37 415 Further, a small cluster of proteins involved in protein folding, including a member of the heat
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39 416 shock protein HSP70 family (GRMZM2G415007/PLAZA identifier ZM04G41380), three
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41 417 chaperone proteins belonging to the Clp protease family (GRMZM2G110023/PLAZA
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43 418 identifier ZM01G09650; GRMZM2G123922/PLAZA identifier ZM10G15640;
44
45 419 GRMZM2G162968/ PLAZA identifier ZM09G19730) and the subunit β of the chaperonin
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47 420 containing T-complex (AC215201.3_FG005/PLAZA identifier ZM06G23100), was
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49 421 identified. It is known that the control of protein folding state is crucial for the survival of
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51 422 plants during abiotic stress (68).
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3 424 *The maize leaf phosphoproteome under drought stress*
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426 Ti^{4+} IMAC enrichment of maize phosphopeptides led to the detection of a total of 980 unique
427 phosphosites on 686 phosphopeptides, which could be mapped on 536 phosphoproteins
428 (**Supplementary Table S10**). The data from the replicate experiments show quantitative
429 reproducibility with an average Pearson correlation of 0.887 and 0.856 for control and
430 drought samples, respectively (**Supplementary Figure S3**). The number of identified
431 phosphoproteins lies in between the range published in recent maize phosphoproteomics
432 studies: 282 phosphoproteins in (23), 858 in (9), 2,852 in (14) and 3,557 in (17). Important to
433 note is that the latter two studies fractionated the enriched phosphopeptides via extensive SCX
434 chromatography, hereby greatly increasing MS analysis time per sample to two days (14, 17).
435 In our work, six hours analysis time was used per sample, hence yielding a relative high
436 number of phosphoprotein identifications.

437 Overall, the majority (97.4%) was mono-phosphorylated peptides, while around 2.6%
438 of the phosphopeptides carried two phosphorylated residues. There were 84.0%
439 phosphoserine, 15.2% phosphothreonine and 0.8% phosphotyrosine containing peptides
440 identified, sharing a similar distribution pattern to other maize phosphoproteomics studies (9,
441 14, 17, 23). All the identified maize phosphosites were searched against the retrieved set of
442 phosphosites identified in maize seed from the Atlas of Maize Proteotypes and a dataset of
443 phosphosites garnered from different developmental zones of maize leaves (17, 38). This
444 resulted in 359 phosphosites (37% of the dataset) uniquely identified in our study
445 (**Supplementary Table S11**).

446 Overrepresentation of amino acid motifs surrounding the identified phosphosites were
447 analyzed using Motif-X (**Table 1**). Phosphorylated tyrosine sites were excluded from the
448 analysis due to their low abundance in the dataset. Similarly to other studies in *Arabidopsis*

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3 449 and other monocots (69-72), [sP] is the most enriched motif for the S-phosphorylation as well
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5 450 as its phosphorylated threonine counterpart [tP] for the T-phosphorylation dataset. Further, 20
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7 451 peptides are enriched with the proline-rich motif [sxSP]. Peptides containing the proline-
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9 452 directed [sP] and [tP] motifs are suggested to be substrates for MAP-kinases (MAPK),
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11 453 sucrose non-fermenting1-related protein kinase 2 (SnRK2), receptor-like kinases (RLKs),
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13 454 AGC family protein kinases PKA, PKG and PKC, CDKs (cyclin-dependent kinases),
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15 455 calcium-dependent protein kinases (CDPKs) and STE20-like kinases (SLKs) (69). Only one
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17 456 common acidic motif – [sDxE] – resulted from the analysis, belonging to 22 peptides that
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19 457 might be potential substrates for casein kinase II (CKII) and CDPKs. Further, three basophilic
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21 458 motifs are overrepresented in the dataset, [Rxxs] and the subtype [RSxs], which are
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23 459 recognized by MAPK kinases (MKKs), and [Kxxs], which is targeted by PKA and PKC. No
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25 460 specific protein kinases are found for the T-phosphorylation motif [tS].
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461

462 *Differential analysis of phosphorylation sites*

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464 Earlier differential phosphoproteomics of maize leaf tissue, identifying differences between
465 stress conditions, lack normalization to the protein abundance (9, 15, 23). Here, thanks to our
466 extensive analysis, we can simultaneously take into account protein and phosphorylation site
467 profiles.

468 In total, 615 phosphosites on 445 phosphoproteins were quantified, of which 536
469 phosphosites in at least two biological replicates of one condition. Taking into account that
470 differences in protein levels can influence the outcome of the differential phosphorylation
471 data, we set out to normalize the intensities of the phosphopeptides to the protein intensities.
472 For 224 phosphosites, matching proteins were quantified in the proteome experiment allowing
473 normalization. This result demonstrated that phosphopeptide enrichment facilitated the

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3 474 identification of low abundance proteins, of which non-phosphorylated peptides were likely
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5 475 missed in the proteome scans due to different dynamic ranges and crowdedness. A two
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7 476 sample test ($p < 0.05$) on the normalized phosphosites intensities showed that 18 of those were
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9 477 differentially regulated by drought (**Figure 2B and Supplementary Table S12**). Some of
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11 478 those phosphosites, S470 on HISTONE DEACETYLASE 6 (GRMZM2G457889) (8.3-fold
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13 479 down regulated upon drought) and S247 stem-specific protein TSJT1 (GRMZM2G169671)
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15 480 (2.5-fold up regulated upon drought), are mapped on proteins previously shown to be
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17 481 regulated during drought signaling (73, 74). In mammalian systems, phosphorylation of
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19 482 HISTONE DEACETYLASE 6 was shown to correlate with enzyme activity and consequent
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21 483 tubulin deacetylation and microtubule destabilization (75). A similar mechanism could take
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23 484 place during drought signaling, as plant microtubules are known to function as sensors for
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25 485 abiotic stress (76).

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29 486 As protein levels for many phosphosites could not be inferred, because no non-
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31 487 phosphorylated peptides of the corresponding proteins were detected, we also subjected the
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33 488 not-normalized phosphosites dataset to a two sample test ($p < 0.05$). Based on the
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35 489 phosphoproteome data alone, we found 44 phosphosites to be statistically significant between
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37 490 two conditions, of which 32 were up-regulated upon drought stress and 12 down-regulated
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39 491 (**Figure 2C and Supplementary Table S13-14**). Interestingly, four microtubule-associated
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41 492 proteins were found to be differentially phosphorylated upon drought stress:
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43 493 MICROTUBULE-ASSOCIATED PROTEIN 70-2 (GRMZM2G017525), DYNEIN LIGHT
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45 494 CHAIN 1 (GRMZM2G472231), MICROTUBULE-ASSOCIATED PROTEIN
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47 495 (GRMZM2G026309), and KINESIN-LIKE PROTEIN KIN12A (GRMZM2G034828).
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49 496 Microtubule-associated proteins are involved in microtubuli organization and their binding
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51 497 affinity to microtubules is known to be controlled via phosphorylation (77, 78). Furthermore,
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53 498 the phosphorylation status of a putative MAP kinase superfamily protein
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3 499 (GRMZM2G044557) and a PROTEIN PHOSPHATASE 2C 64 (GRMZM2G107565;
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5 500 GRMZM2G021610) changed upon water deprivation. In stressed conditions, a SPS1-related
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7 501 proline-alanine-rich protein kinase (GRMZM2G413544; GRMZM2G073399) was found to
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9 502 be less phosphorylated. This serine/threonine kinase is a part of the Sterile 20 (Ste20)-related
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11 503 kinase family that is conserved across the fungi, plant and animal kingdom (79) and its
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13 504 mammalian homologs are known to act as a mediator of stress-activated signals (80). Up to
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15 505 our knowledge this protein has not been related to drought stress.

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18 506 Because of the small dataset of significantly regulated phosphosites, no GO classes
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20 507 showed a significant over- or underrepresentation versus the dataset of all identified
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22 508 phosphorylation sites ($p < 0.05$). Alternatively, we investigated which GO classes were only
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24 509 enriched in the control or the drought stressed samples versus a background set of all maize
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26 510 proteins. This analysis showed that pathways involved in sodium transport, immune response
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28 511 and chromatin silencing are exclusively overrepresented in the drought samples
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30 512 (**Supplementary Table S15**).

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34 514 *A crop PTM database*

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38 516 Comprehensive information of *Arabidopsis* protein phosphorylation sites can be found at the
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40 517 PhosPHAt database (37) and P3DB (81). However, information on detected phosphorylation
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42 518 sites in crop species is only scarcely available in these resources. A very specific database,
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44 519 Atlas of Maize Proteotypes, holds proteomics data for maize seed tissue and can also be
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46 520 consulted to query phosphorylation sites, but only in limited, seed-specific datasets (38). To
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48 521 serve as a general tool for PTMs in all plants, we generated a searchable database for plant
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50 522 protein posttranslational modifications, called *Plant PTM viewer*
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52 523 (bioinformatics.psb.ugent.be/webtools/ptm_viewer/). This database will function as an

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3 524 important resource for future functional studies of plant protein posttranslational
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5 525 modifications, including protein phosphorylation.
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10 527 **CONCLUSIONS**
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14 529 Phosphoproteomics workflows traditionally involve tedious labeling and fractionation steps
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16 530 for comprehensive quantitative analysis of phosphorylation profiles. Here, we present a
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18 531 streamlined and reproducible platform for quantitative phosphoproteomics, which (1) does not
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20 532 require specialized equipment nor expert software and can be easily implemented in any
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22 533 molecular biology lab with access to a mass spectrometer, (2) involves limited sample prep
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24 534 time due to the lack of labeling, fractionation and gel steps, and requires relatively low
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26 535 amounts of starting material [which varies a lot across studies (17, 82, 83)] needed because of
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28 536 the straightforward and sensitive pipeline, (3) does not require excessive MS analysis time as
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30 537 the samples are not fractionated into multiple fractions, and (4) can be applied to model
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32 538 plants, such as *A. thaliana*, and economically important crops, such as maize. Wherever
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34 539 possible, protein levels inferred from non-phosphorylated peptides should be used to
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36 540 normalize phosphopeptide intensities, so that true phosphorylation events rather than changes
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38 541 in phosphoprotein amounts can be monitored. This has so far been poorly implemented in
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40 542 plant phosphoproteomics, but is absolutely essential when reporting differential changes in the
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42 543 phosphoproteome.
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47 544 As a consequence of climate changes, drought stress has become a severe limiting
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49 545 factor in plant growth and productivity throughout the world (84, 85). Showcased in drought-
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51 546 stressed maize leaves, our workflow enabled the in-depth quantitative comparison of
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53 547 phosphorylation patterns. Finally, the data generated, comprising novel (phospho)protein
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55 548 candidates implicated in drought stress signaling, contributes to our understanding of the
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3 549 molecular and cellular mechanisms utilized by crops to survive unfavorable environmental
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5 550 conditions.

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10 552 **ASSOCIATED CONTENT**

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14 554 **SUPPORTING INFORMATION**

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16 555 Figure S1. Pearson correlation coefficient for Arabidopsis proteome and phosphoproteome
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18 556 data.

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21 557 Figure S2. Pearson correlation coefficient for maize proteome data.

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23 558 Figure S3. Pearson correlation coefficient for maize phosphoproteome data.

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25 559 Table S1. Pubmed search.

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27 560 Table S2. Identified proteins in whole proteome analysis in Arabidopsis root

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29 561 Table S3. GO categorization in Arabidopsis proteome dataset

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31 562 Table S4. Total phosphosites identified in Arabidopsis roots

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33 563 Table S5. List of phosphosites in Arabidopsis uniquely identified in this study

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35 564 Table S6. Identified proteins in whole proteome analysis in maize leaves under drought and
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37 565 control conditions

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40 566 Table S7. Maize proteins after filtering and Student's t-test

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42 567 Table S8. Significantly different protein groups upon drought stress in maize leaves ($p < 0.05$).

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44 568 Table S9. GO enrichment analysis of significantly differential maize proteins, with all
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46 569 identified maize proteins as background dataset

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49 570 Table S10. Total phosphosites identified in maize leaves under control and drought conditions

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51 571 Table S11. List of phosphosites in maize leaves uniquely identified in this study

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54 572 Table S12. Significantly different phosphosites upon drought stress in maize leaves ($p < 0.05$),
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56 573 after normalization to protein levels.

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3 574 Table S13. Maize phosphosites after filtering and Student's t-test

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5 575 Table S14. Significantly different phosphosites upon drought stress in maize leaves ($p < 0.05$),
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7 576 without normalization to protein levels.

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9 577 Table S15. GO classes exclusively overrepresented in one of the two samples, either control
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11 578 or drought, with the whole maize annotated genome as background set.

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16 580 **AUTHOR INFORMATION**

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26
27 585 **Author Contributions**

28
29 586 E.S., D.I., K.G., and I.D.S. designed research; E.S., L.D.V., M.V.B, H.N. and F.C. performed
30
31 587 research; E.S. and L.D.V. analyzed data; E.S., L.D.V., K.G., and I.D.S. wrote the paper. All
32
33 588 authors have given approval to the final version of the manuscript.

34
35 589 # or * These authors contributed equally.

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38
39 591 **Notes**

40
41 592 The authors declare no competing financial interest.

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3 875 **FIGURE LEGENDS**

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7 877 **Figure 1. Workflow and key improved steps.**

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11 879 **Figure 2. Differential (phospho)proteome response upon drought stress in maize leaves.**

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14 880 Heat map showing average log₂ values of MaxLFQ intensities of the significantly
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16 881 differentially expressed proteins (A), phosphosite intensities of the significantly regulated
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18 882 phosphosites after normalization to protein levels (B), or phosphosite intensities of the
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20 883 significantly regulated phosphosites without correction for protein abundance (C). The log₂
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22 884 values of the intensities were Z-scored for graphical representation.

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27 886 **Figure 3. Protein-protein interaction networks resulted from STRING analysis of maize**

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29 887 **proteins with significant abundance changes.** Cytoscape was used for visualization.

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32 888 Subnetworks with fewer than 6 interactors are excluded from the representation. Nodes in red
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34 889 represent upregulated proteins, in green downregulated proteins upon drought. Interaction
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36 890 groups are indicated with black circles. The PLAZA identifiers for maize proteins were used
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38 891 because of space constraints.

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900 TABLES

Motif	Motif score	Foreground matches	Foreground size	Background matches	Background size	Fold Increase
.....S.....	16.00	210	784	63261	975876	4.13
.....S.SP...	13.24	20	415	6516	850139	6.29
.....SD.E...	19.40	22	437	3450	853589	12.46
... <td>16.00</td> <td>98</td> <td>535</td> <td>52077</td> <td>905666</td> <td>3.19</td>	16.00	98	535	52077	905666	3.19
...RS.S.....	22.98	39	574	6949	912615	8.92
...K..S.....	6.33	43	395	39892	843623	2.30
.....TP.....	6.60	27	140	35595	556423	3.01
.....TS.....	6.01	29	113	50857	520828	2.63

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902 **Table 1.** Motif-X analysis for overrepresented phosphorylation motifs of all identified
 903 phosphosites in maize leaves.

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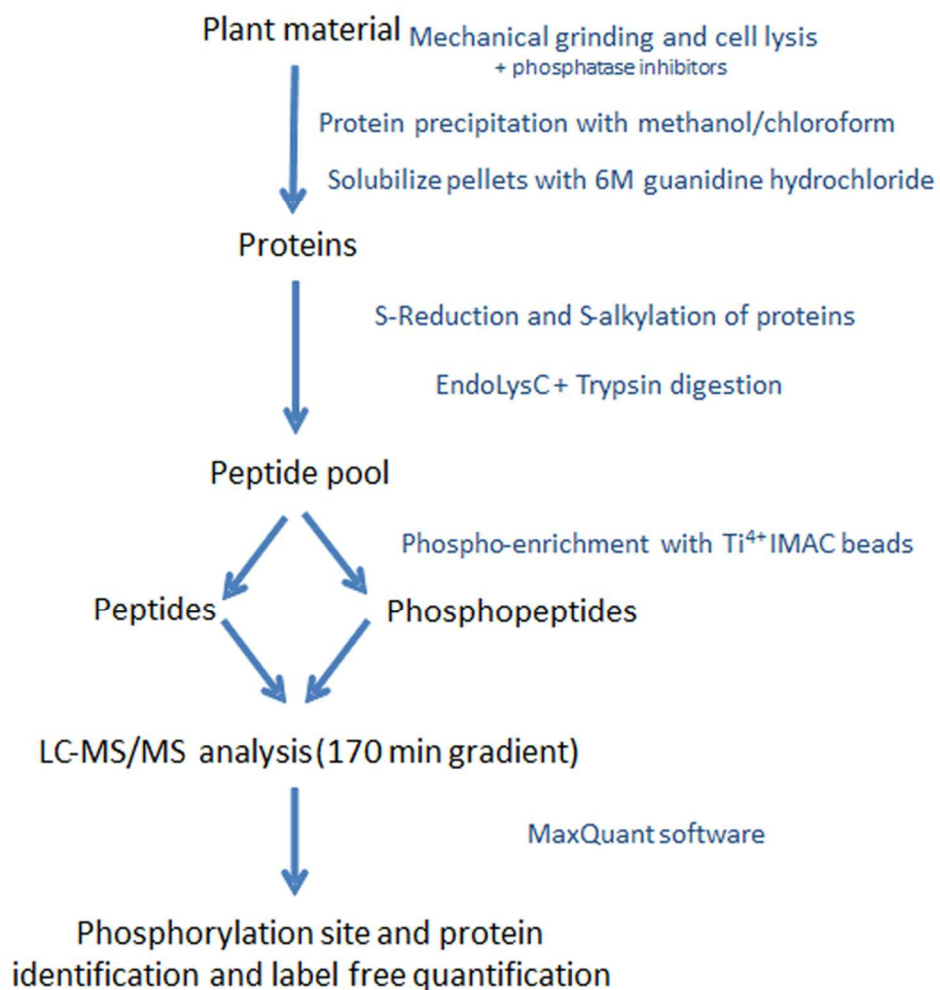


Figure 1. Workflow and key improved steps.
76x78mm (300 x 300 DPI)

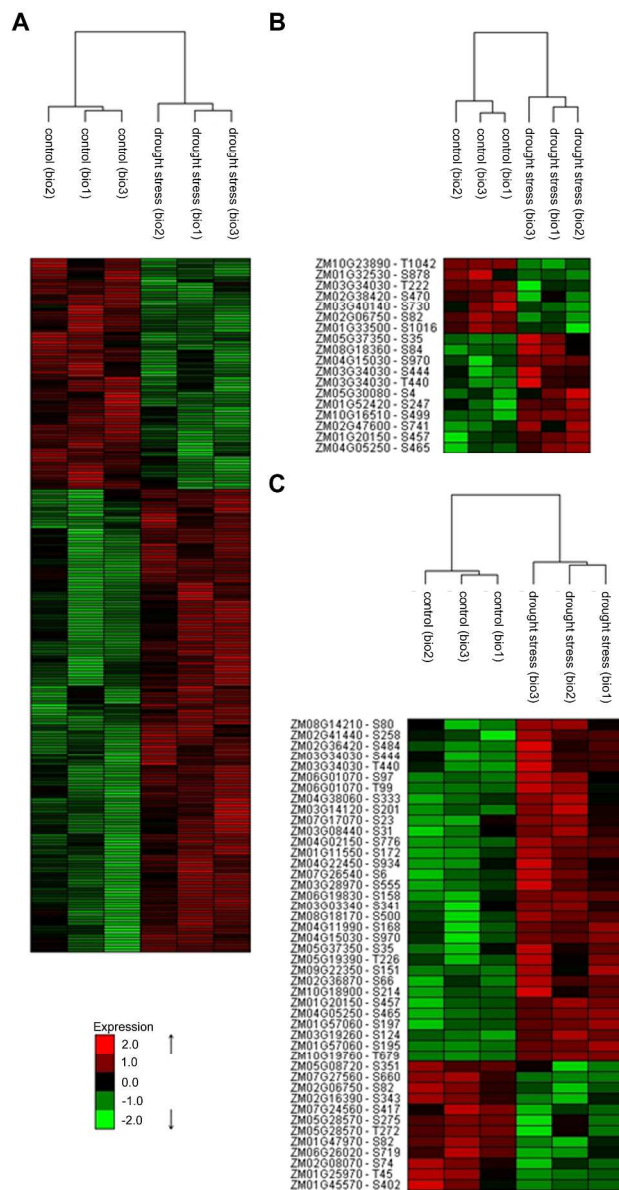


Figure 2. Differential (phospho)proteome response upon drought stress in maize leaves. Heat map showing average log₂ values of MaxLFQ intensities of the significantly differentially expressed proteins (A), phosphosite intensities of the significantly regulated phosphosites after normalization to protein levels (B), or phosphosite intensities of the significantly regulated phosphosites without correction for protein abundance (C). The log₂ values of the intensities were Z-scored for graphical representation.

195x366mm (300 x 300 DPI)

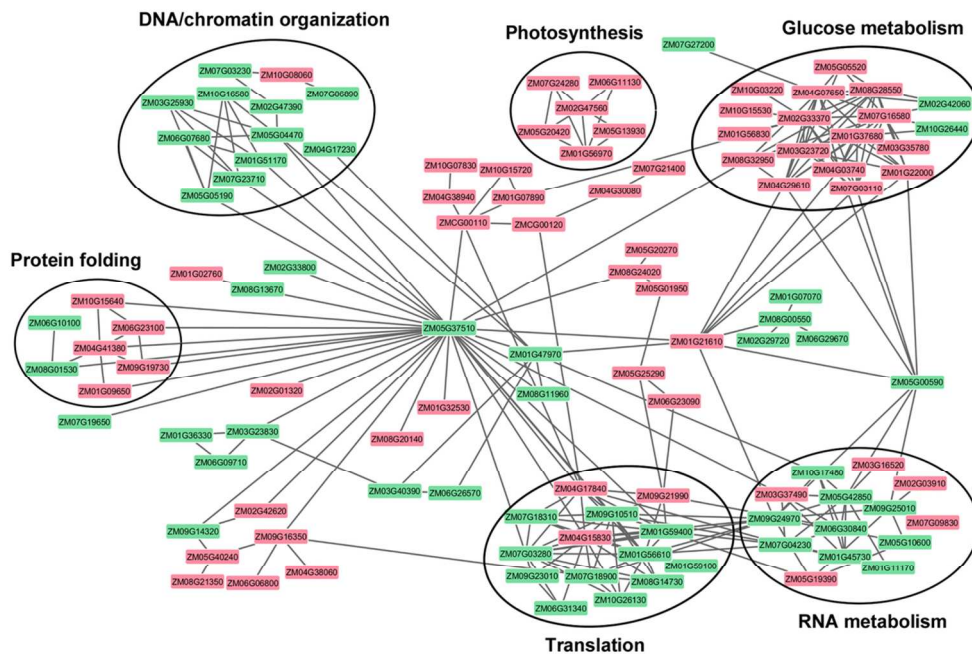


Figure 3. Protein-protein interaction networks resulted from STRING analysis of maize proteins with significant abundance changes. Cytoscape was used for visualization. Subnetworks with fewer than 6 interactors are excluded from the representation. Nodes in red represent upregulated proteins, in green downregulated proteins upon drought. Interaction groups are indicated with black circles. The PLAZA identifiers for maize proteins were used because of space constraints.
106x70mm (300 x 300 DPI)