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Original Article

A metabolomic study in oats (*Avena sativa*) highlights a drought tolerance mechanism based upon salicylate signalling pathways and the modulation of carbon, antioxidant and photo-oxidative metabolism

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ABSTRACT

Although a wealth of information is available on the induction of one or several drought-related responses in different species, little is known of how their timing, modulation and crucially integration influence drought tolerance. Based upon metabolomic changes in oat (*Avena sativa* L.), we have defined key processes involved in drought tolerance. During a time course of increasing water deficit, metabolites from leaf samples were profiled using direct infusion–electrospray mass spectroscopy (DI-ESI-MS) and high-performance liquid chromatography (HPLC) ESI-MS/MS and analysed using principal component analysis (PCA) and discriminant function analysis (DFA). The involvement of metabolite pathways was confirmed through targeted assays of key metabolites and physiological experiments. We demonstrate an early accumulation of salicylic acid (SA) influencing stomatal opening, photorespiration and antioxidant defences before any change in the relative water content. These changes are likely to maintain plant water status, with any photoinhibitory effect being counteracted by an efficient antioxidant capacity, thereby representing an integrated mechanism of drought tolerance in oats. We also discuss these changes in relation to those engaged at later points, consequence of the different water status in susceptible and resistant genotypes.

Key-words: metabolomics; photorespiration; salicylic acid.

Abbreviations: DFA, discriminant function analysis; DI-ESI-MS, direct infusion–electrospray mass spectroscopy; F_v/F_m, the ratio of variable fluorescence (F_v) over the maximum fluorescence (F_m); *m/z*, mass-to-charge ratio. Indicate the dimensionless quantity formed by dividing the ratio of the mass of an ion to the unified atomic mass unit, by its charge number (regardless of sign). To make easier manuscript reading, 'm/z' has been replaced in some places by the word 'metabolite'; PCA, principal component analysis; ROS, reactive oxygen species; SA, salicylic acid.

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INTRODUCTION

Predictions by the Intergovernmental Panel on Climate Change (IPCC) vary from region to region but suggest that many areas would exhibit increased temperatures and severe summer droughts in the near future. Other regions would experience episodes of intense rainfall in winter and autumn, leading to flooding, soil erosion and loss of nutrients (Kumar 2007). Defining the mechanisms of environmental stress tolerance may help secure crop production by targeting traits and genetic loci that could be exploited in plant breeding programmes.

Oat (*Avena sativa* L.) crop is attracting increasing interest as it is well adapted to a wide range of soil types and because it can perform better than other small-grain cereals on marginal soils (Stevens *et al.* 2004). However, oats can be sensitive to hot, dry weather, and hence, in most of the Mediterranean and similar climatic regions, drought is the main limiting factor for yield (Stevens *et al.* 2004). Crop breeders are responding to the challenge of developing new drought-tolerant lines. As in most crops, this is achieved by selection of appropriate progeny. However, for selection of complex traits such as stress tolerance, breeding programmes must be based upon a sound understanding of innate tolerance mechanisms (Blum 1999; Dita *et al.* 2006). When deriving drought-tolerant crop plants, breeders should focus not only on plant survival but also on yield (Turner 1979). This latter aspect is frequently given limited consideration, meaning that drought-tolerant lines often have considerable yield penalties under non-stressful conditions (Passioura 2002). Nevertheless, selection on the basis of yield is not appropriate due to the low heritability of this trait and a high genotype × environment interaction (Araus *et al.* 2002). This is supported by our recent studies on screening of high-yielding oat cultivars for drought tolerance (Sánchez-Martín *et al.* 2012, 2014). Consequently, modern breeding strategies

should attempt to include assessments of physiological, biochemical and molecular characteristics (Araus 1996; Richards 1996; Slafer & Araus 1998), which provide a better understanding of the intricate processes underlying the tolerance response (McWilliam 1989).

Investigations based upon model plants have led to a dramatic increased understanding of the molecular basis of drought tolerance. Several hundred genes that respond to drought stress at transcriptional level have been identified (Seki *et al*. 2002; Yamaguchi-Shinozaki & Shinozaki 2006). Other targeted molecules include transcription factors (Fujita *et al*. 2005), protein kinases (Agrawal *et al*. 2003; Umezawa *et al*. 2004; Osakabe *et al*. 2005; Mori *et al*. 2006) and dehydrin proteins that may aid in stabilizing proteins (Rorat 2006). However, previous work in *Lotus* showed that only a small fraction of metabolites induced under drought were common between the model and the cultivated species, demonstrating the difficult translation of the information derived from model plants to crops. This underlines the importance of studying crops themselves (Sánchez *et al*. 2011; Rasmussen *et al*. 2012).

Biochemical responses of crops associated with tolerance to drought are linked to changes in the metabolic pathways, leading to production of sugars (e.g. from the raffinose family oligosaccharides), sugar alcohols (e.g. mannitol), amino acids (e.g. proline) and amines (e.g. glycine, betaine and polyamines) (reviewed in Seki *et al*. 2007). These metabolites function as (1) osmolytes to reduce cellular dehydration; (2) solutes that stabilize enzymes, membranes and other cellular components; and (3) chelating agents that sequester metals and inorganic ions (Guy *et al*. 2008). These metabolic changes in response to drought reflect changes in photosynthesis (Boyer 1970; Cornic & Fresneau 2002; Lawlor & Cornic 2002; Scheibe 2004) or the activation of futile cycles to prevent over-reduction of photosynthetic electron transport chain components, including those contributing to the photorespiratory cycle and the malate valve (Asada 1999; Cornic & Fresneau 2002; Scheibe 2004). In addition, chemical signalling also influences growth, the timing of reproduction and stomatal function (Davies *et al*. 2002; Sharp 2002; Morison *et al*. 2008). However, little is known about how these responses are integrated to promote tolerance or which responses confer tolerance as opposed to those that are related to susceptibility. Additionally, wider appreciation of the relevance of timing of responses and sensitivity of the plant is needed (Bray 2004; Skirycz *et al*. 2011). Thus, reproducible and thoroughly documented experiments that compare resistant and susceptible genotypes within a species are needed to robustly evaluate genotypic responses to various levels of water deficit (Aguirrezabal *et al*. 2006; Hummel *et al*. 2010). There is also the need to have comprehensive 'omic' level measures of drought stress responses and tolerance mechanisms. Given the importance of biochemical changes in the responses of plants to drought, metabolomic approaches would appear to be particularly appropriate in elucidating tolerance mechanisms.

Metabolomics is an important level of 'omic' analysis (Fiehn 2001, 2002; Hall *et al*. 2002) that allows high-throughput processing of samples with large numbers of replicates so that statistically robust descriptions of a given phenomenon may be produced (Fiehn 2001; Goodacre *et al*. 2004). Further, as the metabolome reflects the summation of transcriptomic, post-transcriptomic and allosteric control of biochemical pathways, it may represent the most accurate description of a given biological phenomenon. In this present study, we carried out a metabolite profiling approach by employing DI-ESI-MS as a tool for the unbiased assessment of metabolic changes in oat leaves in response to drought stress in well-characterized resistant and susceptible oat genotypes. Based upon these studies, we propose an integrated model for drought tolerance in oats, which highlights the relevance of the timing and fine modulation of the responses.

MATERIALS AND METHODS

Plant material, growth conditions and sampling

All experiments were carried out with the oat cultivars (cvs) Flega and Patones, which are susceptible and tolerant to drought stress, respectively (Sánchez-Martín *et al*. 2012). Patones, which exhibits a good adaptation to Mediterranean agroclimatic conditions, was developed by 'Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario' (IMIDRA, Madrid, Spain), and 'Plant Genetic Resources Center' (INIA, Madrid, Spain) provided the seeds. Flega was developed by the Cereal Institute (Thenru- 4
Thessaloniki, Greece). Details of the genetic relationships between these cultivars have been previously reported in Montilla-Bascón *et al*. (2013) and show that they are not closely related.

Experiments were carried out at seedling stage (3-week-old plants) (Xiao *et al*. 2007; Hao *et al*. 2009; Gong *et al*. 2010; Sánchez-Martín *et al*. 2012). Seedlings were grown in 0.5 L pots filled with peat : sand (3:1) in a growth chamber at 20 °C, 65% relative humidity and under 12 h dark/12 h light with 250 μmol m^{-2} s^{-1} photon flux density supplied by white fluo- 5
rescent tubes (OSRAM). During growth, trays carrying the pots were watered regularly. At day 21, water was withheld from those plants selected for drought treatment (Hao *et al*. 2009; Gong *et al*. 2010) for a period of 18 d. Control plants were watered as described earlier throughout the whole experiment. During the drought treatment, the relative water content (RWC) of the soil was monitored daily, reaching a level of approximately 15–20% by day 18 (Gong *et al*. 2010).

At set time points 6, 9, 12, 15 and 18 d, the second leaf of each oat plant from the different cultivars and treatments was harvested from watered and droughted plants, rapidly frozen in liquid nitrogen and lyophilized for metabolomic studies. Each sample consisted of a pool of four leaves with six replicates per time point, cultivar and treatment. After sampling, plants were discarded.

Visual assessment of drought symptoms

Assessment of drought symptoms was carried out on 10 replicates per genotype/treatment according to Sánchez-Martín

et al. (2012). Briefly, drought severity values were assessed daily according to a 0–5 scale, where 0 = vigorous plant, with no leaves showing drought symptoms; 1 = one or two leaves (older leaves) show slight drought symptoms in the tips (less turgor) but most leaves remain erect; 2 = several leaves show a slight decrease in the turgor; however, most of the leaves still show no drought symptoms; 3 = leaves show bending of the tip, although the rest of the leaf remain turgid, incipient yellowing of the older leaf; 4 = all leaves show drought symptoms including incipient wilting and/or yellowing of the older leaf; 5 = all leaves start to appear rolled and/or shrunken (Supporting Information Fig. S1). These data were used to calculate the area under the drought progress curve (AUDPC) similarly to the area under the disease progress curve widely used in disease screenings (Jeger & Viljanen-Rollinson 2001) using the formula:

$$\mathrm{AUDPC} = \sum_{k}^{i=1} \frac{1}{2}[(S_i + S_{i+1})(t_{i+1} - t_i)],$$

where S_i is the drought severity at assessment date i, t_i is the number of days after the first observation on assessment date i and k is the number of successive observations.

RWC

RWC was measured in seven replicates per genotype/treatment according to Barrs & Weatherley (1962). Briefly, 6 h after the onset of the light period, leaf blade segments were weighed (fresh weight; FW), floated on distilled water at 4 °C overnight and weighed again (turgid weight; TW). They were then dried at 80 °C for 48 h. After this, the dry weight (DW) was determined. RWC was then calculated as [6] $\mathrm{RWC} = (\mathrm{FW} - \mathrm{DW})\ (\mathrm{TW} - \mathrm{DW})^{-1} \times 100$.

Cell membrane stability

Cell membrane stability (CMS) was measured in seven replicates per genotype/treatment according to Tripathy *et al.* (2000). Measurements were carried out on the second leaves [7] with a conductivity meter (CMD 510; WPA, UK).

Stomatal conductance

Leaf water conductance (g_l) was measured in 10 plants per cultivar with an AP4 cycling porometer (Delta-T Devices Ltd, Cambridge, UK) according to Prats *et al.* (2006). Measurements were carried out in the second leaves 3 h after the onset of the light period. The measurements were carried out randomly and were finished within 60 min for the set of 80 plants.

Exogenous application of SA

To assess the effects of SA (2-hydroxybenzoic acid), a 100 μM SA solution with 0.1% Tween 20 was sprayed over the entire plant until surface runoff was observed. Control plants were similarly treated with 100 μM of the biologically inactive isomer 4-hydroxybenzoic acid (4-hBA). Treatment was first applied at the initiation of drought treatment and then daily between 3 and 4 h after the onset of the light period until the experiment was completed. Ten replicates were used and measured between day 5 and day 12 after withholding water.

Glyoxylate measurements

Glyoxylate concentration was determined following the method of Rojano-Delgado *et al.* (2010), with slight modifications. Second leaves (250 mg) were ground in liquid nitrogen and homogenized with 1 mL of pure water. Samples were then extracted by ultrasonication for 10 min at 20 °C. The extract was centrifuged at 10 000 *g* for 2 min at 4 °C and two 400 μL of aliquots were obtained. Then, 45 μL of a freshly prepared 1% (w/v) solution of phenylhydrazine in 100 mM HCl was added to each aliquot, and those were kept at 60 °C for 10 min. Afterwards, 225 μL of 1 M HCl was added to acidify the extract and finally 90 μL of potassium ferricyanide (1.6% w/v) was added to one of the aliquots (test aliquot) and 100 μL of pure water to the other aliquot (blank aliquot). Absorbance readings at 532 nm of both aliquots were recorded after 10 min in an HT Synergy microplate reader (Biotek). The glyoxylate content in the samples was calculated as the difference between both readings. [8]

Ascorbate and dehydroascorbate measurements

Ascorbate (AA) and dehydroascorbate (DHA) were determined spectrophotometrically in second leaves (five replicates per genotype/treatment) according to Foyer *et al.* (1983). [9] [10]

Glutathione measurements

Reduced (GSH) and oxidized (GSSG) glutathione were measured in second leaves (five replicates by treatment/genotype) according to Rahman *et al.* (2006).

Sugar measurements

Glucose, fructose and sucrose contents were determined by gas chromatography–mass spectrometry (GC-MS) at 9, 12 and 15 d after withdrawing water in five replicates by genotype/treatment. Lyophilized tissue (50 mg), from second leaves, was ground before extraction under stirring at room temperature in 1 mL of ethanol : water (70:30, v/v) for 30 min. The supernatant was recovered by centrifugation at 4400 *g* for 10 min and the process was repeated under the same conditions to achieve an exhaustive extraction. A clear solution was obtained from each cycle, which was mixed and subsequently derived. Trimethylsilyl oximes (TMSO) were prepared as previously described (Sanz *et al.* 2004).

GC-MS analyses were carried out on a 7890 gas chromatograph coupled to a 5975 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA) using helium as the

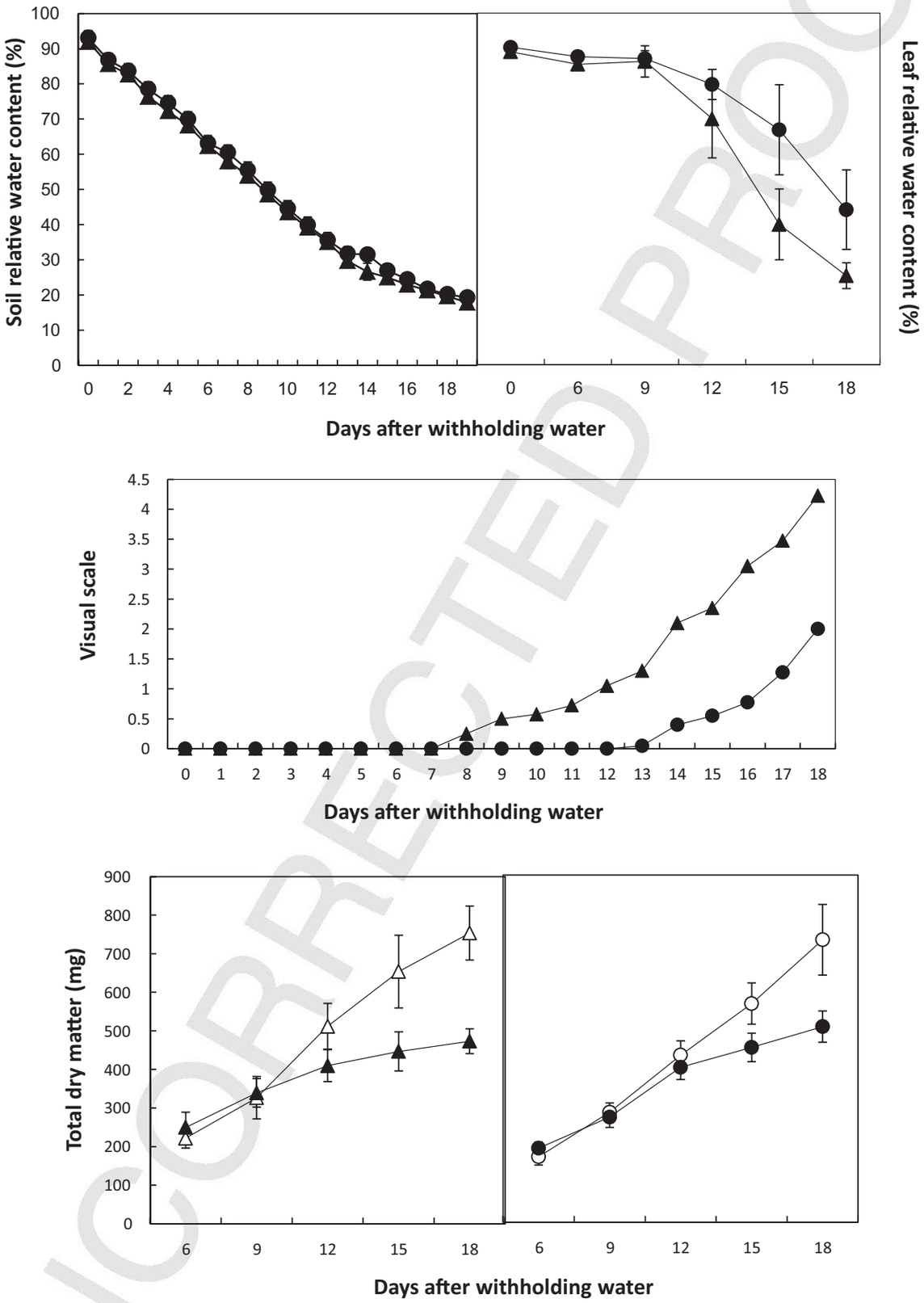

Figure 1. (a) Soil and leaf relative water content; (b) drought symptoms; and (c) total dry matter of Flega (triangles) and Patones (circles) well-watered plants (open symbols), and during a time course of water stress (solid symbols). Data are mean of 10 replicates + SE.

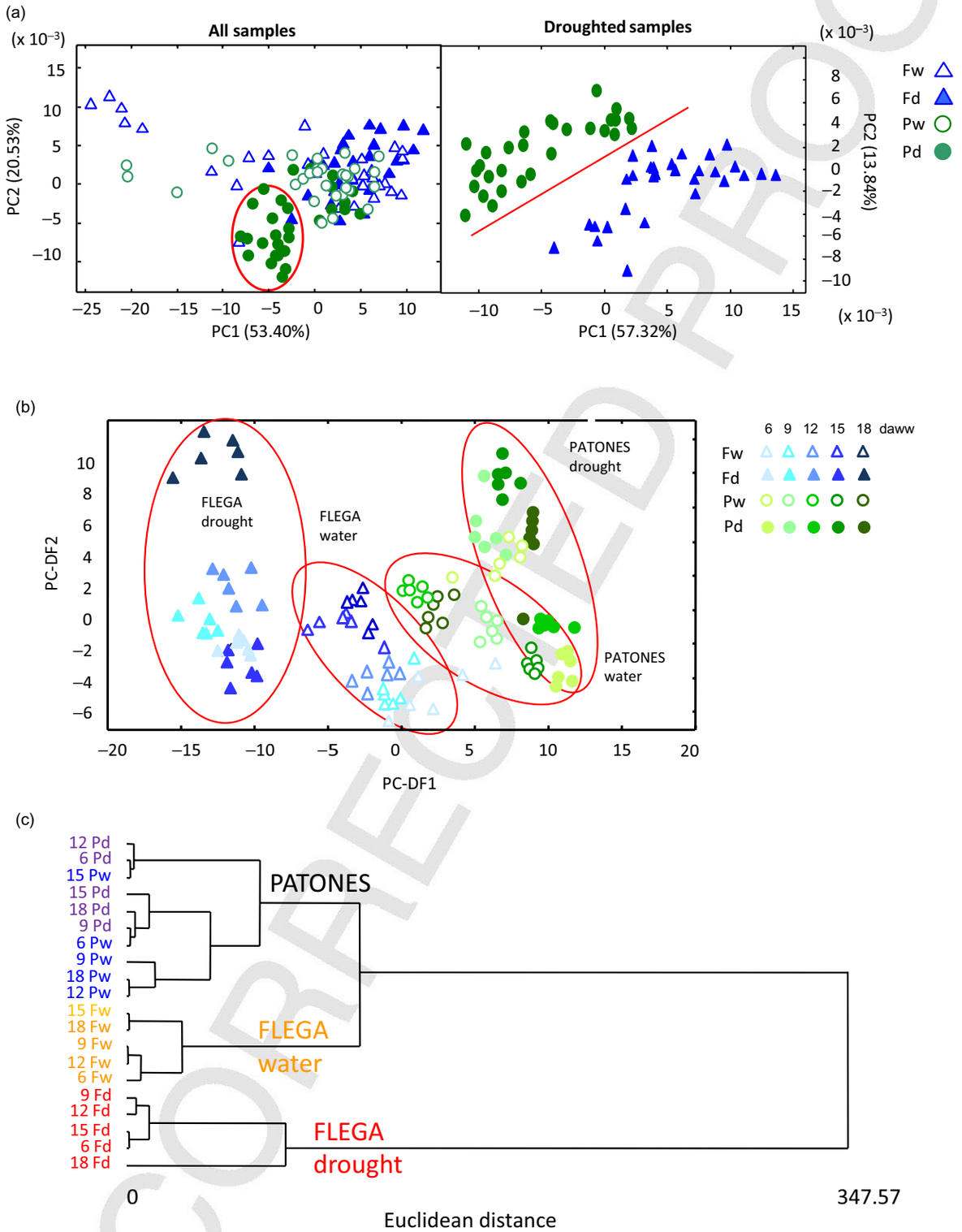

Figure 2. Multivariate analysis of metabolite profiles from oat cultivars Patones and Flega following withdrawal of water. (a) PCA of metabolite profiles with 1398 variables from drought-susceptible Flega (F) and tolerant Patones (P) plants. w = control well-watered plants; d = drought-stressed plants. PCA loadings related to this figure are available in Supporting Information Fig. S6. (b) DFA biplot of PC-DF1 and 2 based upon genotype, treatment and time point classes. The numbers refer to 6, 9, 12, 15 and 18 d after the commencement of the experiment where plants were either regularly watered 'w' or water was withdrawn 'd'. The red ovals group genotypes and treatments and have no mathematical significance. (c) Hierarchical cluster analysis of the selected oat genotypes according to the model represented in (b).

Table 1. Pathways highlighted as discriminating the response of oat cultivars Flega and Patones to water deficit based upon analysis of metabolites from the whole experimental time course

KEGG[a] map	Pathway	Number of metabolites in the pathway	Number of significant metabolites from models	Percentage of significant metabolites
Ko00053	Ascorbate and aldarate	24	15	63*
Map01061	Phenylpropanoids (main core)	20	12	60*
Ko00630	Simplified Glyoxylate/dicarboxylate	12	6	50*
Ko00330	Arginine and proline	36	17	47
Ko00030/40	Pentoses pathway	32	12	38
Ko00250	Alanine, aspartate and glutamate	18	7	38
Ko00052	Galactose	17	6	35
Ko00020	TCA cycle	12	4	33
Ko00051	Fructose and mannose	15	5	33
Ko00500	Starch and sucrose	15	5	33
Ko00480	Glutathione biosynthesis	15	4	27
Ko00270	Cysteine and methionine	37	10	27
Ko00380	Tryptophan	50	13	26
Ko00410	*β*-Alanine	23	6	26
Ko00010	Glycolysis and gluconeogenesis	15	3	20

[a]http://www.genome.jp/kegg/pathway.html.
TCA, tricarboxylic acid cycle.

carrier gas at ~1 mL min^{-1}. A 30 m × 0.25 mm i.d. × 0.25-*μ*m-thick film fused silica column coated with ZB-1MS (Phenomenex, Torrance, CA, USA) was used. The oven temperature was held at 190 °C for 15 min, then programmed to 270 °C at 15 °C min^{-1} and then programmed to 290 °C at 1 °C min^{-1}. The final temperature was held for 10 min. The injector was at 300 °C and injections were made in split mode with a split ratio of 1:20. The mass spectrometer was operated in electronic impact (EI) mode at 70 eV, scanning the 50–650 *m/z* range. The interface and source temperature were 280 and 230 °C, respectively. Acquisition was carried out using HP-ChemStation software (Hewlett-Packard, Palo Alto, CA, USA). Response factors relative to the internal standard were calculated over the expected concentration range. All analyses were carried out in duplicate.

Total salicylate content measurements

Leaf samples were ground in liquid N_2 and extracted overnight at 5 °C in 20 mL of 80% methanol with addition of an internal standard of d6-SA (98 atom %; C/D/N Isotopes, Pointe-Claire, Quebec, Canada). After filtration, methanol was removed by rotary evaporation at 25 °C and samples were centrifuged at 13 400 *g* for 3 min. The fraction was partitioned against an equal volume of ethyl acetate, which was back-washed against H_2O and then reduced to dryness by rotary evaporation at 25 °C. Samples were analysed on a Waters Alliance 2690 liquid chromatograph, using a Waters Nova-Pak C18 cartridge (3.9 mm × 50 mm) (Waters, Milford, MA, USA) eluted at 30 °C at over 15 min with a 10–95% gradient of methanol in 2 mM formic acid at a flow rate of 0.5 mL min^{-1}. One-tenth of the eluate was introduced into a Micromass LCT electrospray ionization mass spectrometer (Micromass/Waters Ltd., UK) operating in a negative ion mode at a sample cone voltage of 30 V, capillary voltage of 2.0 kV and an extraction voltage of 5 V. SA was quantified by calibration of the molar ratio between the $[M - H]^-$ ions at *m/z* 137 (SA) and *m/z* 141 (internal standard).

Chlorophyll fluorescence analysis

Modulated chlorophyll fluorescence was measured on second leaves of dark-adapted plants with a PAM 2100 Fluorometer (Walz, Effeltrich, Germany). Measurements were made on four replicate leaves according to Lichtenthaler *et al.* (2005) and Baker (2008). Dark relaxation kinetics were analysed according to Lichtenthaler *et al.* (2005) to determine the photoinhibitory quenching (q_I) caused by photoinhibition of photosystem II (PSII), a major component of non-photochemical quenching (qN). The chlorophyll fluorescence decrease ratio, R_{fd}, defined as F_d/F_s, was measured using continuous saturating red actinic light for 4–5 min according to Lichtenthaler *et al.* (2005). The irradiance applied for measuring R_{fd} values was checked to avoid photoinhibition of the photosynthetic apparatus. The measurements were carried out at 15 d after withdrawing water in five dark-adapted plants by genotype/treatment.

Proline content

Proline was determined by the ninhydrin method, improved according to Aziz *et al.* (1999) to avoid interference with concentrated sugars. Briefly, lyophilized leaf samples were ground using a ball mill (MM 200; Retsch) with six to eight glass beads (2 mm) in each 2 mL tube. Then, 1.85 mL of distilled water was added. The tubes were kept for 30 min at 100 °C and then cooled to room temperature. The extracts were centrifuged at 4 °C at 3000 *g* for 2 min and the supernatant was placed in a new tube. Then, 250 *μ*L of ninhydrin reagent (1% ninhydrin solution, acetic acid/dH_2O, 60%/40%)

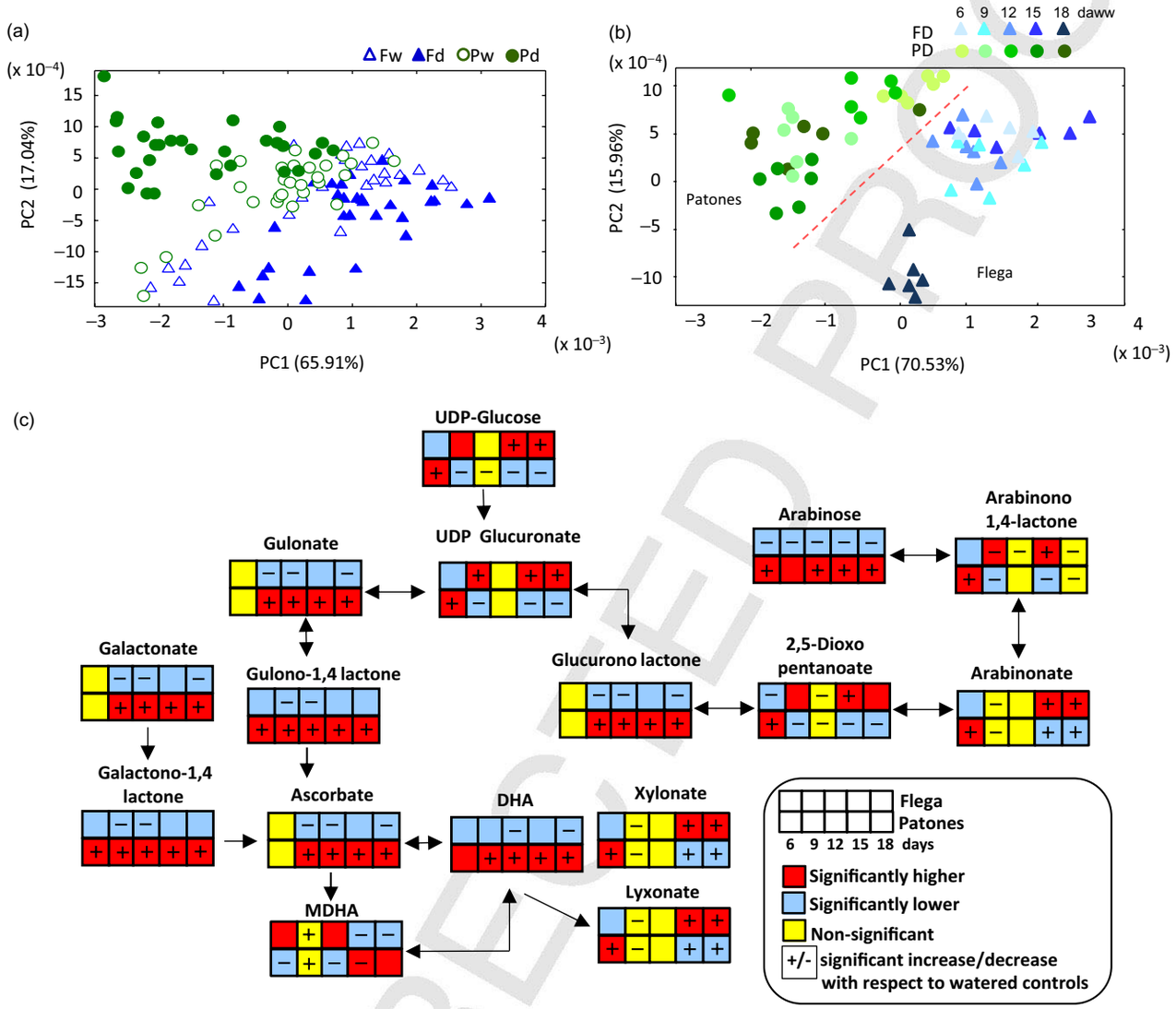

Figure 3. Analysis of metabolites linked to ascorbate pathway in oat cultivars Patones and Flega during a time course of water stress. PCA of metabolite profiles with 24 variables from drought-susceptible Flega 'F' and tolerant Patones 'P' plants (a) comparing drought 'd' and control, well-watered plants 'w' (b), including for analysis only the data corresponding to drought-treated plants classified according to the time exposed to water stress. The numbers refer to 6, 9, 12, 15 and 18 d after the commencement of the experiment. PCA loadings related to (a) and (b) are available in Supporting Information Fig. S6. (c) Heat map of induced changes in significant metabolites linked to the ascorbate pathway in Flega and Patones during the experiment. Red indicates that the differences between droughted and control samples are significantly higher than in the other genotype. Blue indicates that the differences between droughted and control samples are lower than in the other genotype. Yellow indicates no significant differences between genotypes. '+' and '−' within the boxes indicate for a particular genotype and sampling time a significant increase or decrease of the droughted samples with respect to their well-watered controls. Boxes without symbols indicate no significant differences between the droughted and control samples in the corresponding genotype and sampling time.

was added to 50 μL of extract in dark conditions. The mixture was boiled for 30 min and then cooled in an ice-water bath. The chromophore formed was extracted with 500 μL of toluene by vigorous shaking for about 20 s. Absorbance of the resulting organic phase was measured at 520 nm.

DI-ESI-MS and tandem MS-MS

The extraction procedure followed that of Allwood *et al.* (2006). Analysis was carried out using DI-ESI-MS on a Micromass LCT mass spectrometer (Micromass/Waters Ltd) in negative ionization mode where metabolites are singly ionized by the loss of H^+, which has been shown to be effective for characterization of plant extracts (Mattoli *et al.* 2006). The polar extracts were reconstituted in 0.25 mL of 30% (v/v) methanol : H_2O and 50 μL was added to 200 μL inserts in 2 mL (Waters Ltd.) and introduced by direct infusion (DI) at a flow rate of 0.05 mL min^{-1} in 30% (v/v) methanol : H_2O running solvent, using a Harvard 11 syringe pump (Harvard Ltd, Edenbridge, Kent, UK). DI-MS data were

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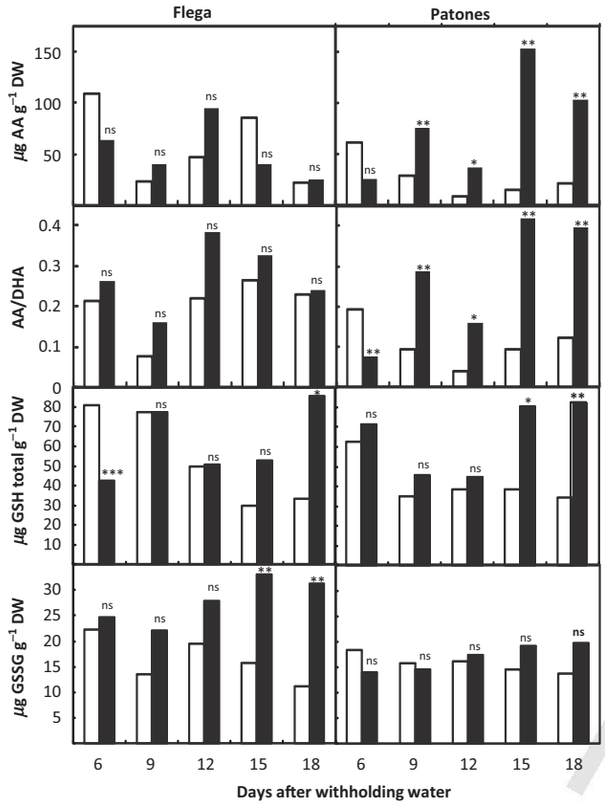

Figure 4. Reduced and oxidized glutathione, ascorbate and dehydroascorbate content. Glutathione [in the reduced (GSH) or oxidized (GSSG] state, ascorbate (AA) and dehydroascorbate (DHA) were quantified in susceptible Flega and tolerant Patones plants during a time course of water stress (6, 9, 12, 15 and 18 d). Data are mean of five replicates. White bar = control, well-watered plants; black bars = plants exposed to water stress. *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, between control and stressed plants; ns indicates no significant differences.

acquired over the *m/z* range of 100–1400 and were imported into MATLAB, binned to unit mass and then normalized to percentage total ion as stated in Johnson *et al.* (2007).

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) using collision-induced dissociation was performed to provide structural information and aid in metabolite confirmation. High-performance liquid chromatography/ion trap mass spectrometry (HPLC/MS^n) analysis was performed on a Thermo Finnigan LC-MS system (Thermo Electron Corporation, USA) comprising a Finnigan Surveyor PDA Plus detector, a Finnigan LTQ linear ion trap with ESI source and a Waters C_{18} reversed-phase Nova-Pak column (4 μm, 3.9 mm × 100 mm). The auto-sampler tray temperature was kept at 5 °C and the column temperature was maintained at 30 °C. Sample injection volume was 10 μL, the detection wavelength was set at 240–400 nm and the flow rate was 1 mL min^{-1}, with 100 μL min^{-1} going to the mass spectrometer. The mobile phase consisted of purified water–formic acid (A; 100:0.1, v/v) and HPLC-grade MeOH–formic acid (B; 100:0.1, v/v). The initial condition was A : B (5:95, v/v), and the percentage of B increased linearly to 60% over 55 min. Mass spectra were acquired in negative ionization mode. Ionization parameters were optimized by infusion of chlorogenic acid standard at a constant rate into the LC flow. MS parameters were as follows: sheath gas 30 arbitrary units, auxiliary gas 15 units, spray voltage 4 kV, capillary temperature 320 °C, capillary voltage −1 V and tube lens offset −68 V. Initial MS/MS fragmentation was carried out at normalized collision energy 35% and isolation width 2.0 (*m/z*).

Data analysis

All experiments were performed in completely randomized designs. For ease of understanding, means of raw percentage data are presented in the tables and figures. However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots to normalize data and stabilize variances, and subjected to ANOVA using SPSS software (SPSS Inc., Chicago, IL, USA), after which residual plots were inspected to confirm data conformed to normality. Shapiro–Wilk test and Bartlett's test was performed to test normality and homogeneity of variances, respectively. Significance of differences between means was determined by least significant difference (LSD) and contrast analysis (Scheffe's).

For multivariate analysis, the data were first analysed using PCA (Causton 1987). PCA and PC-DFA were used as described in Allwood *et al.* (2006) and followed accepted MSI standards (Goodacre *et al.* 2007). All calculations were performed in Pychem 2.0 (Jarvis *et al.* 2006). The analysis identified the key time points and *m/z* to discriminate the susceptible and resistant cvs. Each *m/z* was tentatively identified according to predicted true mass (*m/z* + 1) of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways. Further significant predicted metabolites were grouped according to the biochemical pathways. Finally, multivariate analyses of targeted pathways were performed with only their constituent metabolites to confirm their significance in discriminating drought tolerance responses (Supporting Information Fig. S1). Validation of the PC-DFA models was performed by dividing the data into training and test sets as described by Nicolau & Goodracre (2008).

RESULTS

Physiological responses of susceptible oat cv. Flega compared with tolerant Patones under drought stress

In this work, several independent water stress experiments were conducted to determine the metabolic changes responsible for the drought-tolerant phenotype of cv. Patones compared with the susceptible Flega. For all experiments, the drought treatment imposed on Patones and Flega plants exponentially ($P < 0.001$) reduced the relative water content of the soil (sRWC), reaching approximately 20% with respect to saturated soil at the end of the experiments. Pots with

Flega and Patones followed the same sRWC curve (Fig. 1a), indicating that they were subjected to similar water stress during the whole experiment. Hence, sampling times were chosen to cover different levels of sRWC: still-sufficient water [6 days after withholding water (daww), 55–60% sRWC], mild water deficit (9 daww, 40–45% sRWC), moderate water deficit (12 daww, 30–35% sRWC), high water deficit (15 daww, 20–25% sRWC) and severe water deficit (18 daww; 15–20% sRWC) (Fig. 1a) (Sánchez-Martín *et al*. 2012).

At the beginning of the experiment, plants exhibited a fully emerged second leaf and an incipient third leaf (Supporting Information Fig. S1). Both Flega and Patones showed similar leaf water content at the earlier time points (until 9 daww), but Flega showed a significantly lower leaf water content compared with Patones after 12 daww (Fig. 1a). This was consistent with differences in the visible symptoms of drought (Fig. 1b & Supporting Information Fig. S1). Yellowing symptoms were associated with drought as they were not observed in control, well-watered plants of both Flega and Patones (Supporting Information Fig. S1). When subjected to drought, both Flega and Patones exhibited reduced growth compared with well-watered plants (Fig. 1c), but a similar phenology could be observed between both genotypes and treatments during the experiment (Supporting Information Fig. S1). Thus, by the end of the experiment, control plants showed longer and wider leaves and hence dry matter as compared with the treated ones (Fig. 1c & Supporting Information Fig. S1).

Drought results in more metabolite changes in susceptible oat cv. Flega than in tolerant Patones

Metabolite profiles obtained by DI-ESI-MS and analysed using multivariate approaches determined the genotypic, temporal and treatment-associated responses to drought. Following analysis of all time points and treatments using non-supervised PCAs, clustering into biologically relevant groups was not observed, although the intermediate and late time points of Patones droughted plants tended to differentiate from the rest (Fig. 2a, 'All samples'). Genotype-specific clustering was not observed in PCA of well-watered samples; however, PCA clearly discriminated between cultivars subjected to drought (Fig. 2a, 'Droughted samples'). In addition, application of the supervised DFA approach suggested that the metabolomic responses to drought compared with watered controls were most pronounced in Flega (Fig. 2b). This was supported by hierarchical cluster analysis (Fig. 2c). Cross-validation of data supported the validity of the models proposed.

The PCA loading plots (Supporting Information Fig. S3) showed the relevance of particular variables in the trajectories of the samples. However, given the complexity of the data, in order to resolve metabolite changes in each genotype with different treatments, separate PCA and DFA analyses for each time point were undertaken (an example is shown in Supporting Information Fig. S4 for day 12). Examination of the loading vectors linked to each metabolite revealed key differences at each time point. Those metabolites whose contribution to a given model was greater than ± 1 standard deviation (SD) from the mean value loadings (eigenvalue) were listed as discriminatory metabolites (Lloyd *et al*. 2011; Supporting Information Table S1). Linking tentatively identified metabolites to biochemical pathways can aid in targeting key changes as the constituents of a given pathway are likely to be co-regulated (Lloyd *et al*. 2011). A total of 15 metabolic pathways were highlighted (Table 1). Separate PCAs based only upon metabolites of each particular pathway discriminated between droughted Flega and Patones. The percentage of significant metabolites within each pathway was calculated (Table 1). Three pathways were noted to have more than 50% of significant metabolites discriminating between the responses of Patones and Flega to drought (asterisked in Table 1). These included the ascorbate/aldarate, the glyoxylate/dicarboxylate and the main core of the phenylpropanoid pathway.

Patones exhibits increased antioxidant capacity in response to drought stress

PCA based only upon *m/z* associated with metabolites from the ascorbate pathway proved sufficient to separate the response of Flega and Patones to drought, whereas well-watered controls showed no separation (Fig. 3a & Supporting Information Fig. S5). Further, time-course plots showed distinctive responses in Flega and Patones at different times following withholding of water (Fig. 3b). To compare specific differences in metabolite abundance arising in Flega and Patones as a result of drought conditions, the metabolites contributing to the observed clusters were analysed using two-way ANOVA. Mean values of discriminatory metabolites for watered samples at a given time point were subtracted from those of droughted samples at corresponding time points and displayed as a pathway-associated heat map (Fig. 3c). These results suggested that metabolites linked to AA and DHA exhibited rapid (6–9 d after drought) genotype-specific responses before drought symptoms could be observed. These observations suggested that the AA pool in Flega became more oxidized than in Patones during drought stress.

The importance of the AA changes and other related redox compounds such as glutathione during drought in oats was confirmed through targeted assays of AA/DHA and GSH/GSSG in a repeated drought experiment (Fig. 4). In Flega, the imposition of drought resulted in no change in AA nor in a change in the AA/DHA ratio. In contrast in Patones, there was a significant increase in AA as early as 9 daww with a consequential increase in AA/DHA ratio, which indicated that the total AA pool in Patones was in a more reduced state than that of Flega during drought stress. There was little difference between Patones and Flega in terms of the presence of GSH, both showing some increases towards the end of the drought period. In contrast, the oxidized form (GSSG) was maintained at low, control levels in Patones throughout the experiment, but increased significantly in Flega.

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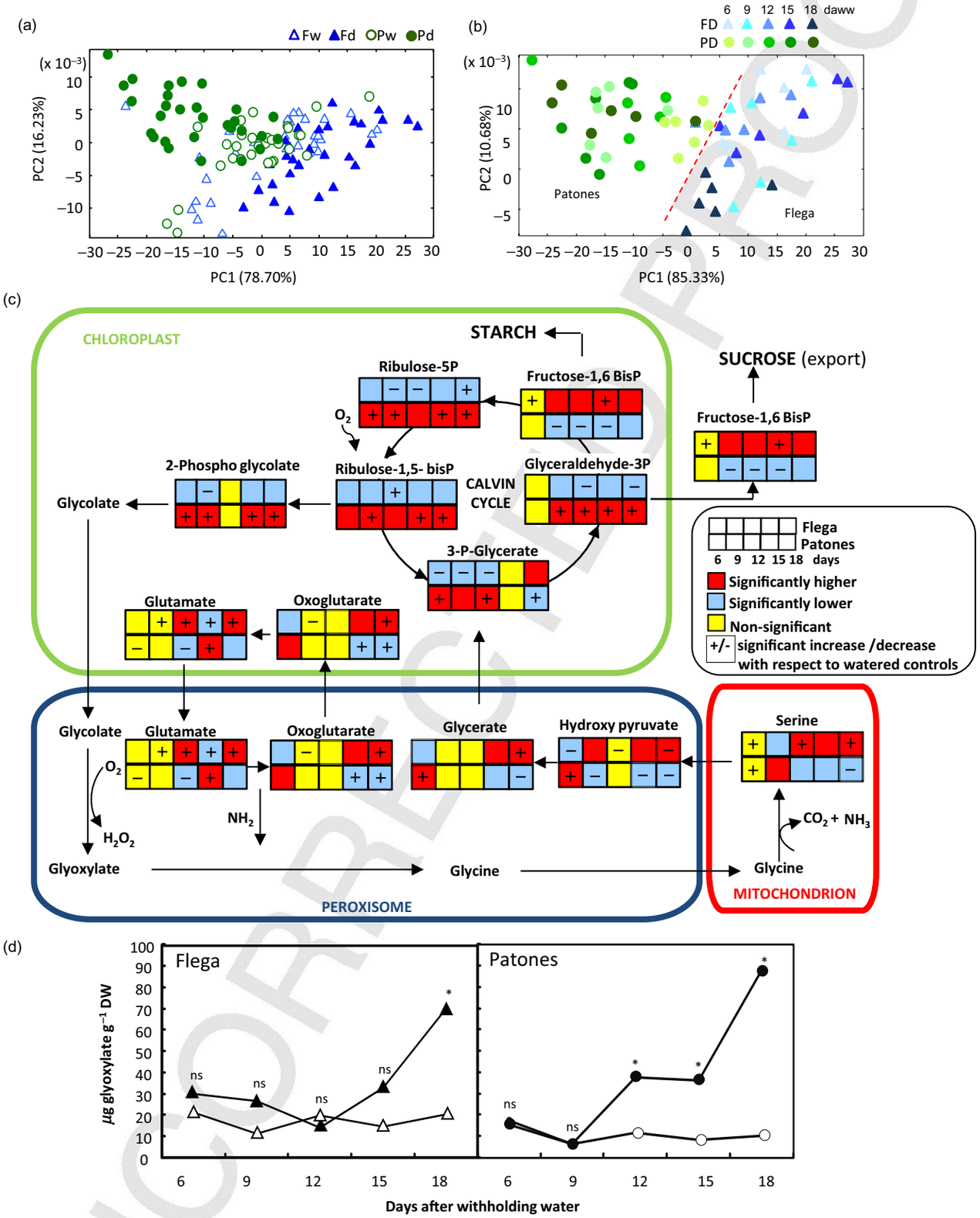

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Figure 5. Analysis of metabolites linked to Calvin cycle pathway in oat cultivars Patones and Flega during a time course of water stress. PCA of metabolite profiles with 10 variables from drought-susceptible Flega 'F' and tolerant Patones 'P' plants (a) comparing drought 'd' and control, well-watered plants 'w' and (b), including for analysis only the data corresponding to drought-treated plants classified according to the time exposed to water stress. The numbers refer to 6, 9, 12, 15 and 18 d after the commencement of the experiment. PCA loadings related to (a) and (b) are available in Supporting Information Fig. S6. (c) Heat map showing changes in significant metabolites linked to the glyoxylate/dicarboxylate pathway in Flega and Patones during the experiment. Red indicates that the differences between droughted and control samples are significantly higher than in the other genotype. Blue indicates that the differences between droughted and control samples are lower than in the other genotype. Yellow indicates no significant differences between genotypes. '+' and '−' within the boxes indicate for a particular genotype and sampling time a significant increase or decrease of the droughted samples with respect to their well-watered controls. Boxes without symbols indicate no significant differences between the droughted and control samples in the corresponding genotype and sampling time. (d) Glyoxylate content in susceptible Flega and tolerant Patones plants during a time course of water stress (6, 9, 12, 15 and 18 d). Data are mean of five replicates. White circles = control, well-watered plants; black circles = plants exposed to water stress. *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, between control and stressed plants; ns indicates no significant differences.

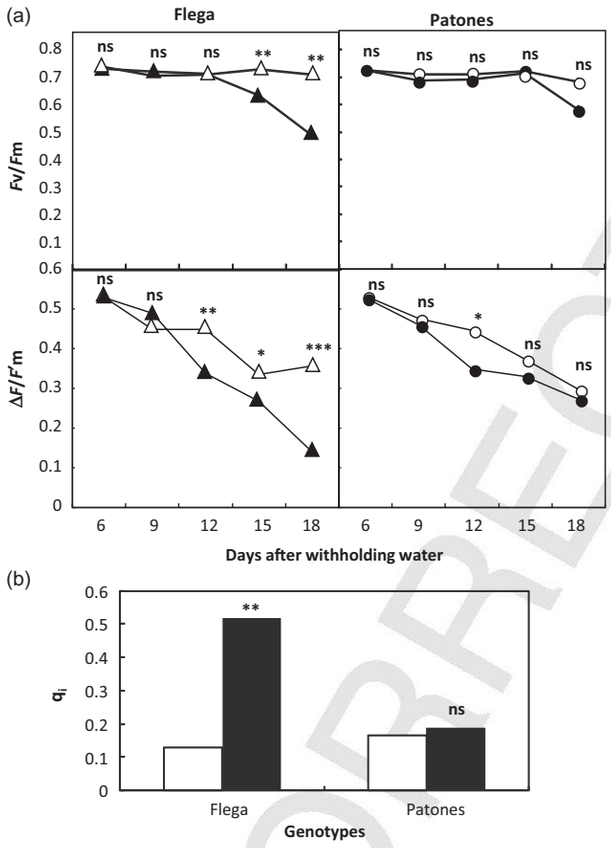

Figure 6. Chlorophyll fluorescence parameters indicative of photosystem II (PSII) photochemistry in susceptible Flega and tolerant Patones plants. (a) Maximum quantum efficiency of PSII photochemistry (F_v/F_m) and operating efficiency of PSII ($\Delta F/F'_m$) in Flega (triangles) and Patones (circles) well-watered plants (open symbols) and during a time course of water stress (solid symbols). (b) Photoinhibitory quenching of PSII in Flega and Patones well-watered plants (white bars) and plants exposed to 18 d of water stress (black bars). Data are mean of four replicates. *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, between control and stressed plants; ns indicates no significant differences.

Metabolites belonging to the photorespiratory pathway are more abundant during drought stress in Patones

PCA based only upon *m/z* associated with metabolites from the photorespiratory pathway proved to be sufficient to distinguish between the responses of Flega and Patones to drought (Fig. 5a,b) but not between watered controls (Supporting Information Fig. S5). These were also analysed using two-way ANOVA (as described earlier for the AA metabolic pathway) and displayed as a heat map (Fig. 5c). This pointed to those metabolites shared with the Calvin cycle as being the most discriminatory, with Patones showing increased abundance compared with Flega. The early (from 6 daww) increased levels of ribulose-1,5-bisphosphate together with 2-phosphogycolate in Patones compared with Flega suggested either a faster formation or slower removal of these metabolites through the pathway. In contrast, in Flega, lower levels of glyceraldehyde-3P and other components of the Calvin cycle were observed (Fig. 5c). In targeted analyses, significant increases in glyoxylate over water controls in Flega were not seen until 18 d after drought (Fig. 5d). In contrast, in Patones, significant increases in glyoxylate compared with water controls were observed from 12 d after drought.

Overall, the metabolomic data indicated a lower antioxidant capacity and photorespiratory activity in Flega, which may lead to increased ROS production and photosynthetic electron flow impairment. To test this hypothesis, the photochemical efficiency of chlorophyll was determined by measuring the maximum quantum yield (F_v/F_m) and operating efficiency of PSII ($\Delta F/F'_m$) (Fig. 6a). F_v/F_m was significantly decreased in Flega from 15 d and $\Delta F/F'_m$ from 12 d following withdrawal of water but Patones was not significantly affected in either parameter within the time frame of the experiment. Photoinhibition of PSII was also assessed at 18 d following withdrawal of water (Fig. 6b) and showed a significant increase in photoinhibition compared with water controls in Flega but not in Patones.

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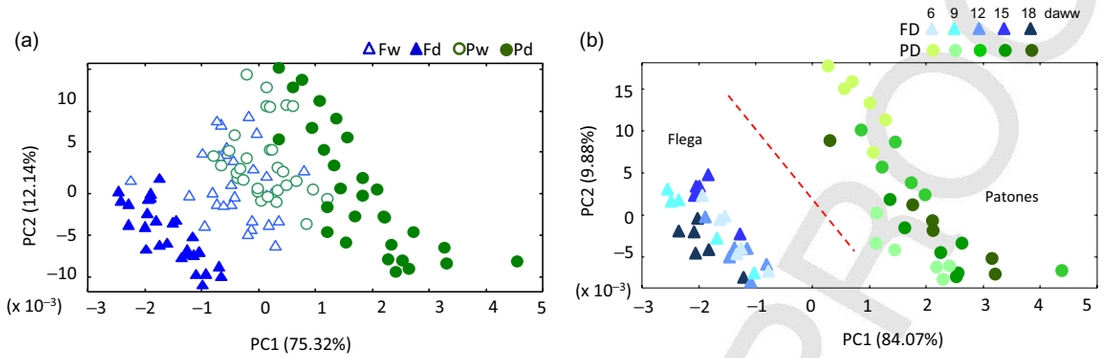

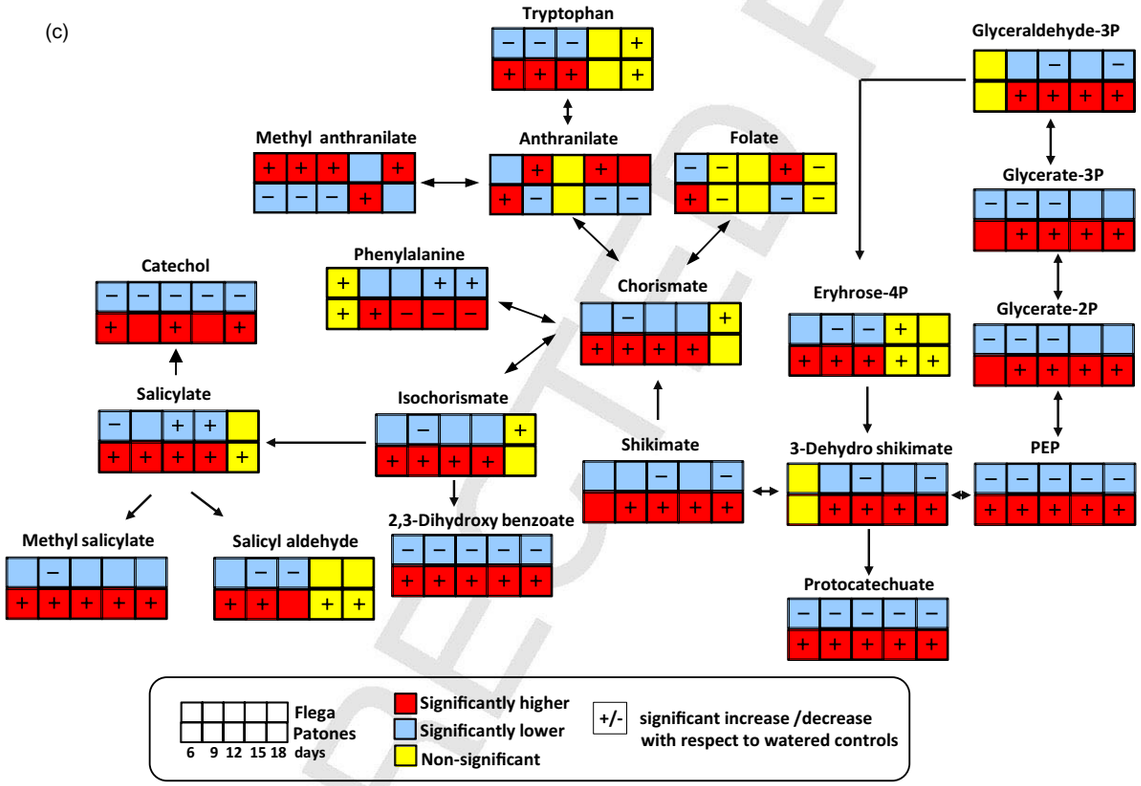

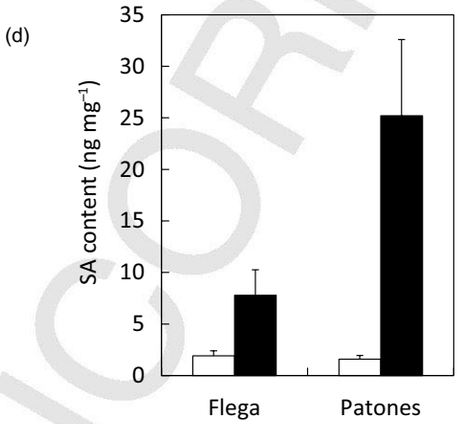

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Figure 7. Analysis of metabolites linked to salicylate metabolism in oat cultivars Patones and Flega during a time course of water stress. PCA of metabolite profiles with 19 variables from susceptible Flega 'F' and tolerant Patones 'P' plants (a) comparing drought 'd' and control, well-watered plants 'w' (b), including for analysis only the data corresponding to drought-treated plants classified according to the time exposed to water stress. The numbers refer to 6, 9, 12, 15 and 18 d after the commencement of the experiment. PCA loadings related to (a) and (b) are available in Supporting Information Fig. S6. (c) Heat map showing significant changes in metabolites linked to the salicylate metabolism in Flega and Patones during the experiment. Red indicates that the differences between droughted and control samples are significantly higher than in the other genotype. Blue indicates that the differences between droughted and control samples are lower than in the other genotype. Yellow indicates no significant differences between genotypes. '+' and '−' within the boxes indicate for a particular genotype and sampling time a significant increase or decrease of the droughted samples with respect to their well-watered controls. Boxes without symbols indicate no significant differences between the droughted and control samples in the corresponding genotype and sampling time. (d) Total salicylate (free and hexose-sugar conjugated) was quantified in susceptible Flega and tolerant Patones plants from control plants (white bar) and at 12 d following the water withdrawal (black bar). Data are mean of five replicates + SE.

Salicylate accumulation in Patones modulates stomatal aperture and delays development of drought symptoms

A major pathway highlighted in Table 1 as potentially discriminating the response of Flega and Patones to drought was that of the phenylpropanoids, specifically targeting shikimate metabolism and important products such as phenylalanine. These are the first metabolites in the core phenylpropanoid pathway and are also involved in the synthesis of the important plant hormone, SA. PCA based upon these phenylpropanoids was unable to distinguish between the well-watered genotypes but readily discriminated between genotypic responses to drought (Fig. 7a & Supporting Information Fig. S5). Examining the latter, changes in Flega were poorly defined over time, but a tendency to cluster between the time points was noted in Patones (Fig. 7b). Heat maps of the shikimate pathway (Fig. 7c) suggested that only in Patones there was an early and continued accumulation of intermediates of the SA biosynthetic pathway such as tryptophan and shikimate, culminating in accumulation of SA (Fig. 7c). The identity of the *m/z* linked to SA was confirmed by tandem MS, and accumulation of SA in Flega and Patones leaves was confirmed by a targeted assay. This indicated that while SA increased in both Flega and Patones under water deficit, the increase was significantly greater ($P < 0.001$) in the latter (Fig. 7d).

To establish that SA could confer drought tolerance to oats, the effects of exogenous application of SA were compared to treatment with a non-biologically active isomer (4-hydroxybenzoic acid). Application of SA was found to delay the development of drought symptoms in Flega and, to a lesser extent, in Patones (Fig. 8a), revealing significant ($P < 0.01$) symptom reductions in SA-treated plants during drought stress in both genotypes.

One mechanism through which SA could promote drought tolerance could be through the promotion of stomatal closure. In both watered-controls and droughted plants, Flega exhibited significantly reduced stomatal conductance in response to SA but did not cause tight stomatal closure. This was not the case with Patones where exogenous SA had no additional effect (Fig. 8b). The area under the conductance progress curve was also calculated for SA-treated plants and controls, confirming that SA significantly reduced stomatal conductance, although only in Flega. In addition, in both genotypes, SA significantly improved RWC at 14 d after withdrawal of water. However, no impact on CMS was noted in either genotype (Fig. 8c).

Carbon assimilate metabolism is maintained in cv Patones during drought while proline increased only in Flega

Besides the three pathways previously considered, Table 1 also highlighted other pathways, most of which were related to carbon metabolism and osmoregulatory pathways. To validate these observations, the main soluble sugars were independently quantified. There was a greater increase in glucose and fructose at intermediate water deficit in the susceptible Flega than in Patones. However, whereas glucose and fructose content drastically declined in Flega at 15 daww, in Patones, these sugars continued to accumulate. Sucrose content significantly decreased in Flega droughted plants compared with controls from 9 daww, whereas this reduction was observed at a later time point in Patones (Fig. 9a). These data could suggest a sustained photosynthetic activity in Patones driving carbon assimilate metabolism, whereas this would be impaired in Flega at high stress dose. To support this hypothesis, the photosynthetic CO_2-fixation rate inferred through R_{fd} measurements (Lichtenthaler *et al.* 2005) was examined. A significant reduction of the net CO_2 assimilation rate was observed in Flega under drought stress but not in Patones (Fig. 9b).

Proline pathway was also associated with drought tolerance and is highlighted in Table 1. Independent quantification of proline (Fig. 10) showed a significant and early (9 daww) increase of this amino acid in Flega plants when subjected to drought stress. Eightfold differences compared with control plants were observed in Flega during the most severe water deficit. Droughted Patones plants showed a significant increase in proline compared with well-watered plants but only at the most severe stress dose (18 daww) (Fig. 10). The distinctiveness of the patterns of proline accumulation compared with those of other amino acids was assessed following targeted analyses. Different patterns to proline were observed for several amino acids (Supporting Information Fig. S6). These observations could not be easily linked to a drought tolerance mechanism in Patones.

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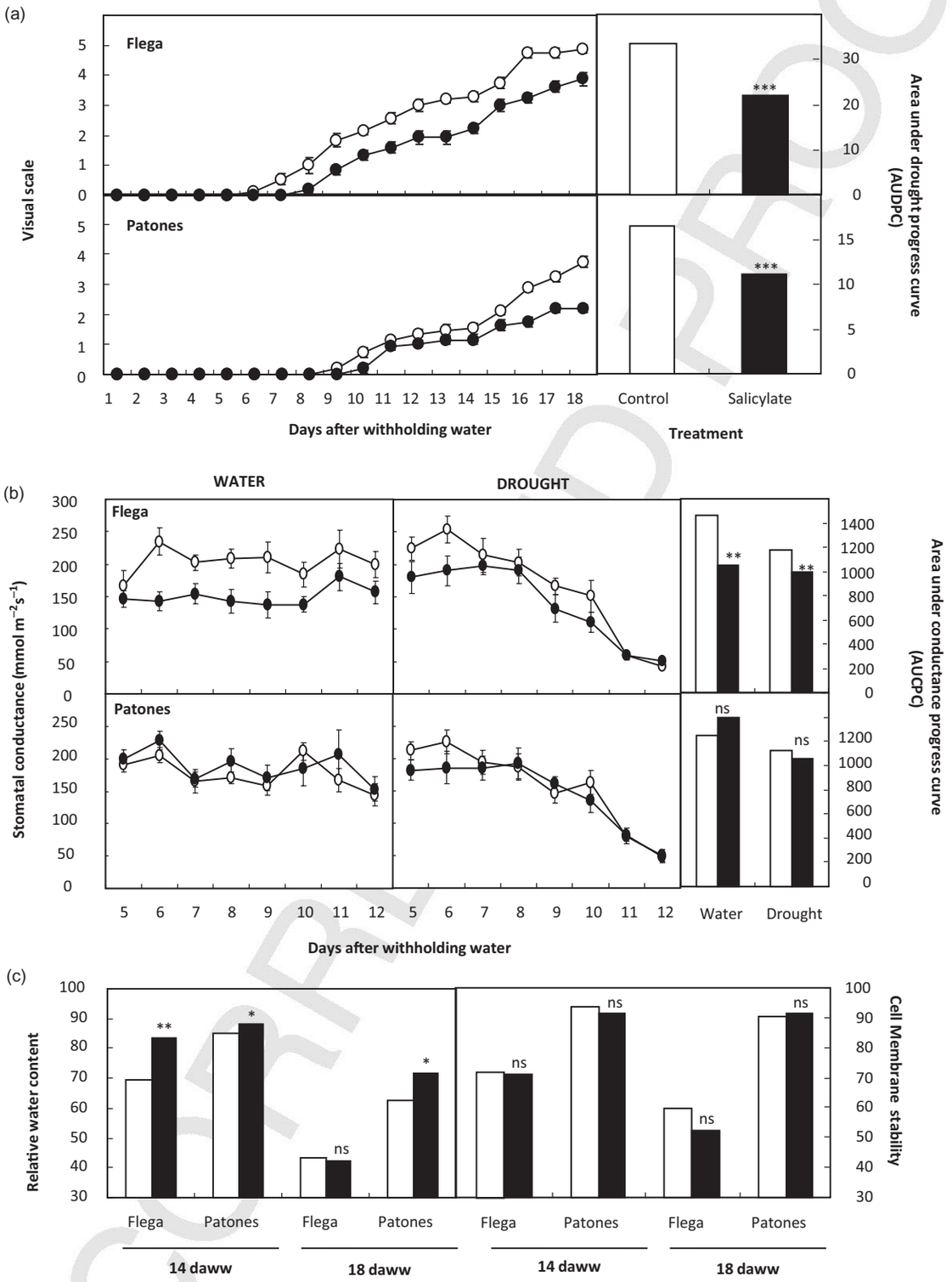

Figure 8. Effect of salicylate on Flega and Patones plants exposed to water stress. (a) Emergence of drought symptoms in Flega and Patones, well-watered and exposed to water stress. White circles = control plants; black circles = salicylate-treated plants. (b) Stomatal conductance of leaves of Flega and Patones, well-watered and exposed to water stress. White circles = control plants; black circles = salicylate-treated plants. (c) Relative water content and cell membrane stability of Flega and Patones plants exposed to water stress. Data are mean of 10 replicates + SE. White bars = control plants; black bars = salicylate treated plants. * and ** indicate significant differences at $P < 0.05$ and $P < 0.001$, respectively, between control and treated plants; ns indicates no significant differences.

DISCUSSION

Because of the cross-species ubiquity of many metabolites, metabolomic studies are particularly well-placed to describe large-scale physiological events in non-genome-verified organisms such as oats. According to our data, genotype-associated variation in the metabolome was not seen under well-watered conditions but only when Flega and Patones were subjected to drought, thereby allowing the identification of the responses induced by drought and linked with a resistant or susceptible phenotype. There was some limited, non-significant discrimination between Patones and Flega controls, most likely reflecting the genetic distance between the genotypes. However, detailed analysis of the specific metabolites under consideration such as the SA showed that the significant differences were only 'set-on' upon water deficit.

We expected that plants would incrementally reprogramme metabolism as sRWC lowered; therefore, we displayed heat maps to suggest changes over time. Notably, a response in terms of ascorbate and associated metabolites occurred earlier in the resistant cv Patones, being observed before any change in leaf RWC is visible, most likely indicating a more sensitive perception of water deficit. As in this present work, early increases in the abundance of antioxidant proteins of the ascorbate and glutathione cycle have been also observed in grapevines exposed to a progressive increase of water deficit (Cramer *et al.* 2013). Both ascorbate and glutathione peroxidase were highly elevated from day 4 and remained elevated compared with controls afterwards. This suggested the importance of the early regulation at different levels of the antioxidant machinery to cope with the water deficit. High levels of reductants (including glutathione) are crucial for the regeneration of ascorbate, which is required for sustained rapid turnover of the water–water cycle and therefore to prevent oxidative damage.

During drought-induced stomatal closure, the maintenance of the light reactions under reduced internal CO_2 concentrations can result in the over-reduction of the photosynthetic components. Thus, photoinhibition is likely to be an essential facet of drought stress in areas such as the Mediterranean (Pfannschmidt *et al.* 2009). Indeed, in an analysis of 11 drought-adapted Mediterranean species, adaptation to low CO_2 was linked to the maintenance of active Rubisco (ribulose 1,5-bisphosphate carboxylase) as an important component for photoprotection (Galmes *et al.* 2011). Our biochemical/biophysical data show that Flega plants subjected to drought

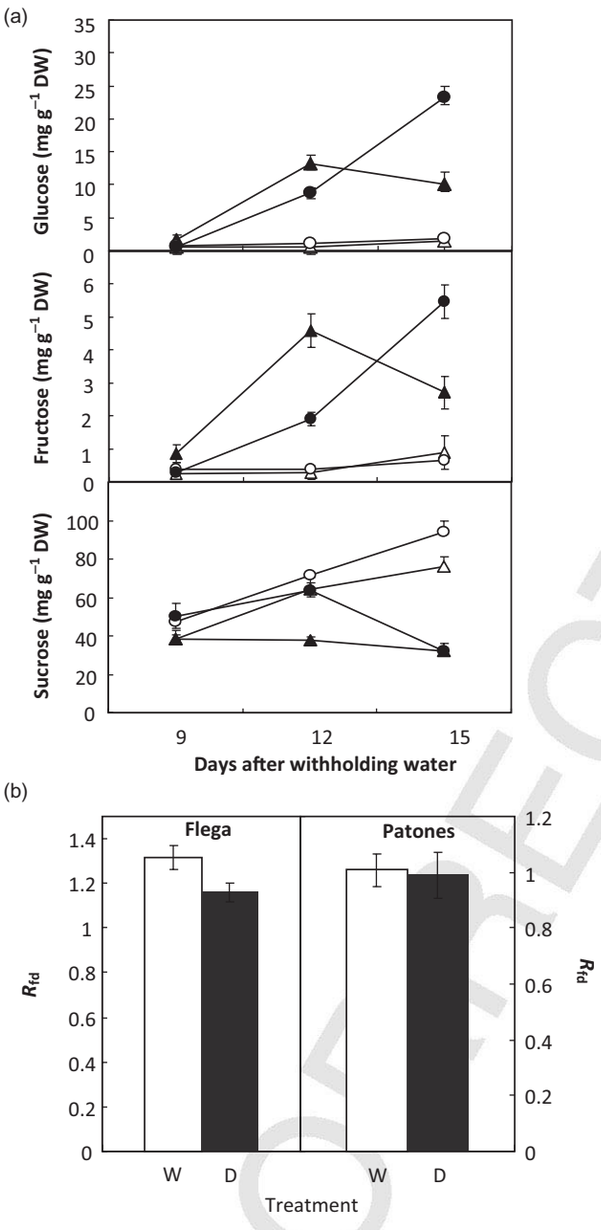

Figure 9. (a) Glucose, fructose and sucrose content in Flega and Patones. Glucose, fructose and sucrose were quantified in drought-susceptible Flega (triangles) and Patones (circles) well-watered plants (open symbols) and during a time course of water stress (solid symbols) (9, 12 and 15 d). (b) Chlorophyll fluorescence decrease ratio (R_{fd}) values indicative of photosynthetic CO_2-fixation rates in Flega and Patones plants at 15 d after withholding water. White bars = control, well-watered plants (W); black bars = plants exposed to water stress (D). Data are mean of five replicates + SE.

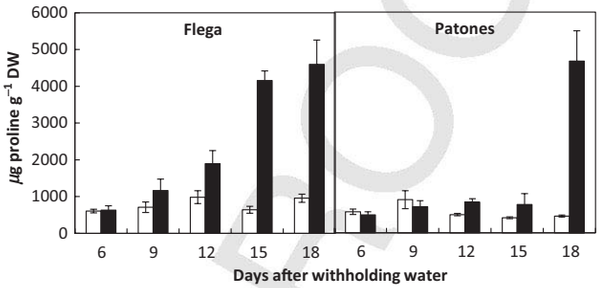

Figure 10. Proline content. Proline was quantified in susceptible Flega and tolerant Patones plants during a time course of water stress (6, 9, 12, 15 and 18 d). White bar = control, well-watered plants; black bars = plants exposed to water stress. Data are mean of five replicates ± SE. *, ** and *** indicate significant differences at $P < 0.05$, 0.01 and 0.001, respectively, between control and stressed plants; ns indicates no significant differences.

dramatically reduced the relative quantum efficiency of electron transport through PSII, showing photoinhibition at the end of the drought time course. There was a higher accumulation of photorespiratory pathway intermediates in Patones, particularly those related to the Calvin cycle, phosphoglycolate and glyoxylate. These increases might be due to increased activities of the Rubisco or glycolate oxidase or to the low activities of the enzymes that recycle these metabolites (Wingler *et al*. 2000). Irrespective of the mechanism, the lower abundance of ribulose bisphosphate in Flega with respect to Patones suggests a lower regeneration of this intermediate which might contribute to the photoinhibition observed in Flega. In previous work (Cramer *et al*. 2013), abundance of photorespiration proteins was observed at both early (i.e. serine hydroxymethyltransferase) and late (i.e. glycolate oxidase and glycine dehydrogenase) stages of water deficit, suggesting the relevance of the regulation at the protein level. In addition, it is known that glyoxylate is an efficient precursor for oxalate biosynthesis (Yu *et al*. 2010). Oxalate may regulate stomatal aperture by binding calcium ions in the vicinity of the guard cells (Ruiz & Mansfield 1994). Thus, it is possible that the increase in glyoxylate might be related to the fine regulation of the stomatal aperture observed in Patones,

A fine-tuning of responses leading to drought tolerance was revealed through our SA experiments. Our observations indicated that the accumulation of endogenous SA was associated with the drought response in Patones. Significantly, SA has recently been reported to induce stomatal closure along with nitric oxide (NO) production in *Arabidopsis* (Khokon *et al*. 2011). Significantly, NO-mediated stomatal closure has been shown to enhance the adaptative response of wheat to drought stress (García-Mata & Lamattina 2001). SA appears to be particularly effective in closing stomata in epidermal strips (Manthe *et al*. 1992; Lee 1998; Mori *et al*. 2001), although *in planta*, SA effects were smaller and needed higher concentrations (Manthe *et al*. 1992). Here, exogenous application of SA reduced stomatal conductance in Flega both under well-watered and drought conditions. Interestingly, SA application had no effect on Patones stomatal conductance, which could indicate that in this cultivar SA could be at or near the maximum threshold at which it exerts its influence on stomatal aperture. Previously, we have observed that under drought, Patones reduces stomatal aperture but maintains the circadian rhythms longer than Flega, which closes stomata early and tightly reduces conductance to dark levels (Sánchez-Martín *et al*. 2012). The retention of low-amplitude opening and closing in response to circadian rhythms in Patones seemingly enabled a delicate equilibrium between saving water and maintenance of photosynthesis (CO_2 fixation) while avoiding oxidative damage. Clearly, this is not only mediated by SA as crosstalk between SA, ABA and other hormones such auxin, ethylene, and jasmonate is also crucial in determining the size of stomatal apertures (Acharya & Assmann 2009). However, it is possible that concentrations of many of these hormones were below the detection limits of the MS approach and so have been missed in our metabolomic screen. In contrast to the fine stomatal modulation in Patones, tight stomatal closure in Flega would result in low internal CO_2 concentration, which would affect photosynthetic activity and add to the increased electron flow-associated oxidative stress. In line with this, our R_{fd} data indicated a decrease in the photosynthetic CO_2 fixation rate in Flega at 15 daww, which was not observed in Patones. In agreement with recent reports (Bogeat-Triboulot *et al*. 2007; Hummel *et al*. 2010), the water deficit at the earliest sampling times did not lead to a down-regulation of carbon metabolism but rather to an increase. According to these authors, this could be due to maintenance of the rate of net CO_2 assimilation and reduction of leaf expansion, which would lead to the significant increases of glucose and/or fructose observed in both Flega and Patones. Alternatively, if photosynthesis is being maintained, it is possible that products accumulate as not being used to drive other processes limited by water supply (protein synthesis, cell division).

It is unlikely that the effect of SA was limited to stomata regulation. SA has been shown to alleviate drought symptoms by maintaining cellular redox homeostasis through regulation of antioxidant enzymes (Durner & Klessig 1995) and induction of the alternative respiratory pathway (Moore *et al*. 2002). It is significant that these pathways were also identified here through metabolite profiling to be important during metabolic adjustment to drought stress. In addition, the biosynthesis of several enzymes of metabolic pathways included in Table 1, such as the glyoxylate cycle, the pentose phosphate pathway, glycolysis and gluconeogenesis, is strongly activated by SA (Rajjou *et al*. 2006), suggesting a complex role for SA in drought tolerance.

Our analyses indicated other pathways that were less prominent in our metabolite profiles. The direct quantification of proline showed an earlier increase of this amino acid in Flega compared with Patones, which correlated with the decrease in leaf RWC. Proline has long been recognized as a marker of drought stress (Barnett & Naylor 1966; Bates *et al*. 1973; Nayyar & Walia 2003; Bowne *et al*. 2012) and has been proposed for selection of drought-tolerant plants (Barnett & Naylor 1966; Bates *et al*. 1973; Nayyar & Walia 2003). Against this, accumulation of proline in rice and sorghum genotypes under salt stress was deemed to be a symptom of damage rather than an indication of tolerance (Ashraf & Foolad 2007). Similarly, our data showed that the increase in proline correlated with the decrease in CMS that occurred earlier in Flega than in Patones. Thus, this increase would be related to the cellular stress instead of increased tolerance.

Taking all of our work together, we suggest an integrated – albeit most likely incomplete – model of the metabolomic changes involved in drought tolerance mechanisms in oat (Fig. 11). Thus, in the tolerant cv Patones, early and fine modulation of stomatal closure involving SA reduces large-scale water losses but avoids photosynthetic impairment and associated increases in ROS. Any ROS produced would be scavenged through induction of the AA-related antioxidant and photorespiratory pathways. In contrast, in Flega rapid and tight stomatal closure together with low and/or later induction of photorespiration and antioxidant pathways could lead to an increase in ROS, damaging the photosynthetic apparatus and decreasing CMS. These rapid metabolic responses in Patones suggest either faster drought sensing or a lower stress threshold required to trigger ameliorative

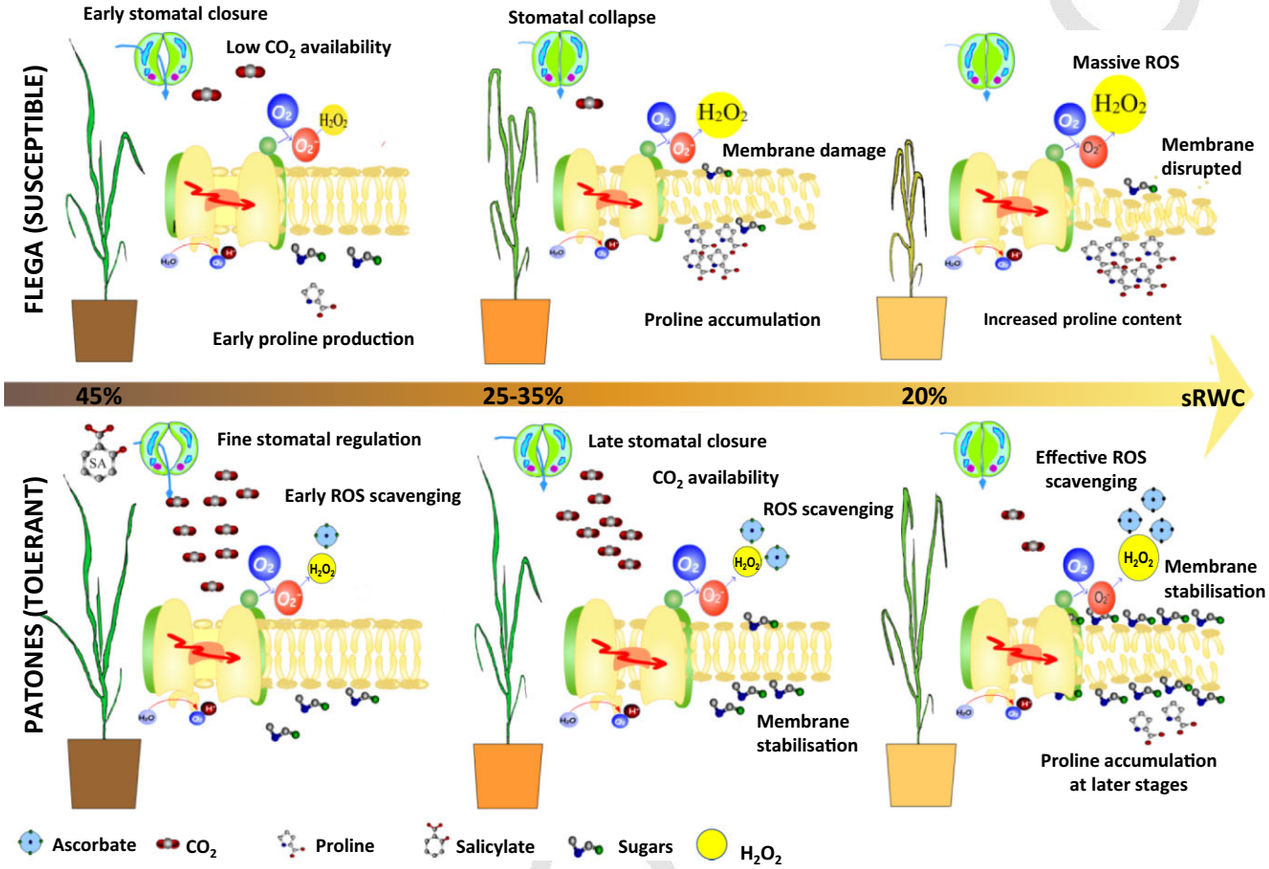

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Figure 11. An integrated model of drought tolerance in oat cultivar Patones as compared to the response in the susceptible cultivar Flega. The schematic brings together the physiological and metabolomic observations described in this paper. In Patones, fine modulation of stomatal closure involving SA reduces large-scale water losses but avoids photosynthetic impairment and therefore increases ROS. Any ROS produced would be scavenged through induction of the AA-related antioxidant and photorespiratory pathways. In contrast, in Flega rapid and tight stomatal closure together with low and/or later induction of photorespiration and antioxidant pathways could lead to a raise of ROS, damaging the photosynthetic apparatus. Early damage of cell membrane in this later genotype would lead to an earlier proline accumulation compared with Patones.

mechanisms. In contrast, the susceptible cv Flega exhibited accumulation of commonly reported drought tolerance markers such as the amino acid proline, which would be related to a survival response in this genotype (Fig. 11). Based upon these models, it is possible to suggest the most appropriate targets – either biochemical or linked genes/markers – which could be exploited in cereal breeding.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Pictures of Flega and Patones plants at key time points of the water deficit time course experiment. [39]

Figure S2. Analysis pipeline.

Figure S3. PCA loadings.

Figure S4. Example sequential analyses of mass spectrometry profiles from a single experimental time point.

Figure S5. Principal component analysis of pathways.

Figure S6. Exemplary metabolic changes in susceptible oat Flega and tolerant Patones under drought.

Table S1. Significant compounds highlighted by the general model (modelled with all residuals) and related metabolic pathways.

Table S2. Significant compounds from the analysis of each of the specific pathways arisen from the general analysis but that did not appeared significant in the general analysis.

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