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The Complex Response of Free and Bound Amino Acids to Water Stress During the Seed Setting Stage in *Arabidopsis*

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SUMMARY

Free and protein-bound amino acids (FAAs and PBAAs) in seeds play an important role in seed desiccation, longevity, and germination. However, the effect that water stress has on these two functional pools, especially when imposed during the crucial seed setting stage is unclear. To better understand these effects, we exposed *Arabidopsis* plants at the seed setting stage to a range of water limitation and water deprivation conditions and then evaluated physiological, metabolic, and proteomic parameters, with special focus on FAAs and PBAAs. We found that in response to severe water limitation, seed yield decreased, while seed weight, FAA and PBAA content per seed increased. Nevertheless, the composition of FAAs and PBAAs remained unaltered. In response to severe water deprivation, however, both seed yield and weight were reduced. In addition, major alterations were observed in both FAA and proteome compositions, which indicated that both osmotic adjustment and proteomic reprogramming occurred in these naturally desiccation-tolerant organs. However, despite the major proteomic alteration, the PBAA composition did not change, suggesting that the proteomic reprogramming was followed by a proteomic rebalancing. The proteomic rebalancing has not been observed previously in response to stress, but its occurrence under stress strongly suggests its natural function. Together, our data show that the dry seed PBAA composition plays a key role in seed fitness and therefore is rigorously maintained even under severe water stress, while the FAA composition is more plastic and adaptable to changing environments, and that both functional pools are distinctly regulated.

INTRODUCTION

Plants have evolved strategies to escape water stress or to tolerate it (Chaves *et al.*, 2002, Verslues and Juenger, 2011). Some species avoid water stress through rapid germination, growth, and early flowering, while other species invest in tolerance by shifting their resource allocation strategy while maintaining metabolic activity (Farooq *et al.*, 2009, Farooq *et al.*, 2014). The extent to which water stress affects plant growth, productivity, and metabolism depends heavily on the genotype, but the timing, intensity, and mode of imposition of the stress as well as the developmental status of the plant are also key factors (Huber *et al.*, 1984, Tardieu *et al.*, 2000, Reymond *et al.*, 2003, Bray, 2004, Hummel *et al.*, 2010, Rethore *et al.*, 2019).

All plant species manifest several morphological, physiological, and metabolic acclimations when faced with water stress. Stomatal closure is one of the most important responses plants have to prevent excess transpiration. This strategy comes at a cost since it leads to reductions in photosynthetic activity, production of photoassimilates, and nitrogen and carbon uptake (Shaner and Boyer, 1976, Sharp and Davies, 1979, Ferrario-Mery *et al.*, 1998, Foyer *et al.*, 1998, Chaves *et al.*, 2002, Farooq *et al.*, 2009, Hummel *et al.*, 2010, Pinheiro and Chaves, 2011, Robredo *et al.*, 2011, Zhong *et al.*, 2017). These metabolic and physiological changes in the vegetative tissues, in turn, can have a profound effect on the reproductive tissues, especially when the response occurs during the seed setting stage. For example, the reduction in photoassimilate production during this stage negatively affects mobilization of carbon and nitrogen to flowers and seed sink tissues (Leport *et al.*, 2006,

Mafakheri *et al.*, 2010, Zare *et al.*, 2012, Sevanto, 2014). Water stress also alters the hormonal balance in the plant, which further inhibits the enzymes and transporters involved in the mobilization of the photoassimilates from the vegetative tissues to the sink tissues (Shaner and Boyer, 1976, Ferrario-Mery *et al.*, 1998, Foyer *et al.*, 1998, Liu *et al.*, 2004, Yang *et al.*, 2006, Urano *et al.*, 2009, Robredo *et al.*, 2011). All these changes can have detrimental effects on gametogenesis, fertilization, embryogenesis, maturation, and production of seed storage reserves (Barnabas *et al.*, 2008, Stagnari *et al.*, 2016, Sehgal *et al.*, 2018), all of which can lead to a reduction in seed yield and weight.

Less clear are the effects of water stress during the seed setting stage on a seed's nitrogen and protein content or overall amino acid levels and composition. Some studies report a reduction in nitrogen and protein content in several legumes (Nayyar *et al.*, 2006, Singh, 2007, Kirnak *et al.*, 2010, Ghanbari *et al.*, 2013). Other studies, however, report an increase in seed proteins in legumes and cereals (Bouchereau *et al.*, 1996a, Gooding *et al.*, 2003b, Teixeira and Pereira, 2007). These inconsistent findings could be due to differences in genotypes, in the water stress imposed, and/or in environmental conditions (Ku *et al.*, 2013, Stagnari *et al.*, 2016). In addition to these quantitative alterations in protein content, water stress also affects the seed's proteomic composition, including altering the absolute level or relative composition of the seed storage proteins (SSPs) (Zhang *et al.*, 2014). These proteins serve as the main storage for nitrogen and carbon in seed and can comprise up to 70% of a seed's total amino acid content (Herman and Larkins, 1999, Don *et al.*, 2006, Nguyen *et al.*, 2015). While these proteins contribute to flour quality and seed longevity and vigor, they are relatively poor in essential amino acids (Li *et al.*, 2018, Ren *et al.*, 2018, Zheng *et al.*, 2018), thus their high abundance negatively affects a seed's overall nutritional quality (Galili and Amir, 2013, Galili *et al.*, 2016). Attempts to reduce SSPs in several model plant systems using multiple approaches has resulted in only minor changes to a seed's overall amino acid composition, a phenomenon known as proteomic rebalancing (Schmidt *et al.*, 2011, Morton *et al.*, 2016). It is not known whether a similar proteomic rebalancing response would be activated when a seed's proteome is altered in response to water stress.

Approximately 90-99% of the total amino acid (TAA) in seeds are protein-bound, and only 1-10% is free (Muehlbauer *et al.*, 1994, Cohen *et al.*, 2014). Despite their relatively low levels, FAAs play important roles in seed development, maturation, and desiccation. They are, first and foremost, the building blocks for proteins. They are also the precursors of many primary and secondary metabolites involved in core metabolic functions as well as in plant protection against biotic and abiotic stresses (Fait *et al.*, 2006, Angelovici *et al.*, 2011, Galili *et al.*, 2016, Xing and Last, 2017). The function of the FAA pool at the end of desiccation is not clear. Several FAAs that accumulate during late maturation are rapidly consumed during early germination,

implying a role in germination (Fait *et al.*, 2006). Other abundant FAAs in seeds, such as proline (Pro), may facilitate proper development and desiccation or function as alternative energy sources (Mattioli *et al.*, 2009, Kishor and Sreenivasulu, 2014, Amir *et al.*, 2018). Levels and composition of FAAs in the vegetative tissues change considerably in response to stress, generally increasing (Hildebrandt *et al.*, 2015, Hildebrandt *et al.*, 2018). These increases are due, mainly, to alterations in the metabolism of the FAAs that act as osmoprotectant or from protein turnover (Sanders and Arndt, 2012, Hildebrandt *et al.*, 2015, Huang and Jander, 2017, Hildebrandt, 2018, Hirota *et al.*, 2018). What effects different intensities or durations of water stress have on the accumulation of FAAs in dry seeds is an open question. This question is especially intriguing since seeds are inherently desiccating tissues with molecular mechanisms that facilitate severe water dehydration (Leprince *et al.*, 2017).

To gain a comprehensive understanding of how water stress effects the two amino acid functional pools, we exposed *Arabidopsis* plants to increasing intensities of water limitation or to increasing durations of water deprivation (drought) regimes during the seed setting stage and then analyzed the physiological, metabolic, and proteomic outcomes in the dry seeds. Our underlying assumption is that the levels and composition of both the FAA and PBAA pools in the dry seed represent the optimum metabolic outcome (Li *et al.*, 2018, Ren *et al.*, 2018) needed to ensure seed longevity, germination, and seedling establishment. We chose the *Arabidopsis* Columbia accession as our model system since it exhibits rapid flowering under dehydration, which alludes to its general drought escape adaptation (Verslues and Juenger, 2011). It also produces few seeds even under harsh conditions, which indicates its ability to allocate sufficient resources to the female generative organs to ensure the continuation of its genetic line (Sun *et al.*, 2004).

RESULTS

Two water stress impositions were applied to *Arabidopsis* plants during the seed setting stage

To examine how *Arabidopsis* seeds respond to water stress imposed during the seed setting stage, we exposed plants to two water stress treatments during seed setting and evaluated key physiological and biochemical traits before proceeding to an in-depth analysis of seed amino acid levels and composition. Our experimental design, which is shown in Figure 1, involved subjecting plants to either a water limitation treatment or a water deprivation treatment starting 1 week after bolting. The water limitation treatments, which were designed to mimic limited rainfalls or controlled irrigations, involved allowing the soil to dehydrate to 10, 25, 50, or 75% pot capacity (PC) and then maintaining that PC condition until the end of seed setting, which occurred at six

weeks after bolting in our control plants, altogether exposing plants to 5 weeks of stress. Plants maintained at 100% PC at all times served as controls (Figure 1a). The water deprivation treatments, which were designed to mimic various durations of terminal drought, involved depriving plants of 0, 1, 2, 3, 4, or 5 weeks of water. Plants deprived of 5 weeks of water (5WS) were the most stressed; plants not deprived of any water (0WS) served as controls (Figure 1b).

To evaluate the stress imposed and perceived by the plants, the relative water content (RWC) of both the soil and leaves was measured throughout the duration of the treatment period. In the water limitation treatments, soil RWC declined gradually until each target PC was reached, which occurred about two weeks after the stress was imposed, i.e., week 3 after bolting (Figure S1a). Plants were able to maintain their leaf RWC at ~88% for all treatments except for the most severe treatment (i.e., 10% PC), which dropped to 54% after 3 weeks of stress (Figure S1b). Both soil RWC and leaf RWC dropped sharply under all water deprivation conditions. In all cases, soil RWC decreased first followed, one week later, by a decrease in leaf RWC (Figure S2a, b). Neither type of water stress had an effect on seed moisture content (Table S1) or germination rate (Table S2). We found no significant differences between control seeds and seeds from plants exposed to either of the most severe stress treatments (i.e., 10% PC and 5WS) for either physiological trait.

Water limitation led to a decrease in seed yield but an increase in seed weight, while water deprivation led to reductions in both traits

Water limitation: Although plants exposed to water limitation were generally able to maintain normal leaf RWC, the plants maintained at 50% PC or less had substantially diminished aboveground tissues as compared to the controls (Figure S3a). Those same plants also demonstrated diminishing seed yields and increasing seed weights: at 50% PC seed yield (defined as the average weight of all seeds recovered per plant) decreased by ~18% and seed weight increased by ~17%, and at 10% PC seed yield decreased by 68% and seed weight increased by 30% (Figure 2a, b). Altogether these plant had fewer, but heavier seeds. Water deprivation: Plants grown under water deprivation conditions also had reductions in their aboveground tissues, starting as early as 2 weeks after the water stress was imposed (Figure S3b), followed by a gradual reduction in seed yield, which decreased by 45% in plants exposed to 2WS to 89% in plants exposed to 5WS (Figure 2c). In addition, seed weight started decreasing gradually from 16% at 3WS to 31% at 5WS (Figure 2d). Plants exposed to just one week of water deprivation had no significant difference in seed yield as compared to controls, which indicates that the seed setting stage was completed at week 5 after bolting under our growth conditions.

Seed nitrogen content has increased while the carbon's remained unaltered in response to both severe water limitation and deprivation when comparing equal sample weights

To evaluate the effect that the various water stress treatments had on nitrogen and carbon content of dry seeds, we measured the levels of nitrogen and carbon from equal sample weights (carbon/nitrogen per mg) and compared the levels across all treatments. Since the physiological analyses showed that seed weight was affected by the type and severity of the water stress imposed, we also calculated and compared the nitrogen and carbon levels in single seeds (carbon/nitrogen per seed) in order to assess changes at the whole-seed level. To further understand the changes at the whole-seed level, Spearman correlation analyses were performed between seed weight and both carbon and nitrogen content per seed.

Water limitation: We found that nitrogen content per mg was significantly elevated (~11%) only in response to the most severe water limitation treatment (10% PC) and that carbon content per mg remained relatively unchanged across all treatments (Figure 3a, b). Calculations of both nitrogen and carbon per seed showed substantial increases in plants maintained at 50% PC or less (Figure S4a, b), which was consistent with a strong positive correlation found between seed weight and both nitrogen and carbon content per seed (Table S3a).
Water deprivation: We found that seed nitrogen content per mg increased by ~13-24% in plants subjected to 3 weeks or more of water deprivation stress and that seed carbon content per mg remained unaltered in response to all water deprivation conditions (Figure 3c, d). In contrast, nitrogen content per seed fluctuated across the treatments, but declined overall when compared to the control (Figure S4c). Carbon content per seed decreased in all treatments, starting 1 week after the stress was imposed (Figure S4d). Consistently, seed weight correlated strongly with carbon content per seed (r=0.87), but only moderately with nitrogen (r=0.55) (Table S3b)

Total lipid content per weight was reduced in response to severe water stress, but the decline was more prominent in response to water deprivation

Before performing an in-depth analysis of the amino acids content in seeds, we evaluated the effects that the most severe water stress conditions had on oil, one of the main storage compounds in *Arabidopsis* seeds. In *Arabidopsis*, almost all of the lipids stored in dry seeds are triacylglycerols (a storage form of oil) (Baud *et al.*, 2002, Li *et al.*, 2006, Baud *et al.*, 2008); therefore, we measured total lipid content per mg and per seed from the 100 (control) and 10% PC treated plants (water limitation) and from the 0 (control) and 4WS treated plants (water deprivation); the 5WS treated plants did not yield enough seed for this analysis.

The total lipid content per mg was reduced in response to both types of severe water stress. However, severe water deprivation had a greater effect than severe water limitation on lipid content: a decline of ~27% versus 3%, respectively (Table S4). Calculating total lipids per seed showed that total lipid content per seed increased by ~26% in response to severe water limitation, but decreased by almost 50% in response to severe water deprivation (Table S4). These observation are consistent with the elevation of seed weight at severe water limitation and the decrease in seed weight at severe water deprivation (Figure 2b, d).

The content of total bound amino acids per weight increased in response to both types of severe water stress

To evaluate the effects that the various water stress treatments had on overall protein levels as well as amino acid composition, we measured PBAA content per mg from dry seeds. We used acid hydrolysis combined with a targeted LC-MS/MS-MRM approach (Data S1a, b). Due to the acid hydrolysis step, tryptophan (Trp) and cysteine (Cys) were lost, asparagine (Asn) was converted to aspartic acid (Asp), and glutamine (Gln) was converted to glutamic acid (Glu); therefore, Asx represents Asn plus Asp, and Glx represents Gln plus Glu. In addition, this step destroys all FAAs, leaving only the amino acids that were part of the protein structure (PBAAs). Glycine (Gly) was excluded due to poor reproducibility. In the end, the total number of amino acids quantified was 15, representing 17 amino acids. We calculated total PBAA (TPBAA) content per mg (nmol/mg) as well as relative content per seed (nmol/seed). TPBAA content per mg was figured by summing all 15 PBAAs quantified (Figure 4a, c, Data S1a, b). TPBAA content per seed was figured by summing the absolute levels of each of the 15 PBAAs measured per seed (Figure S5; Data S1c, d). Thus, TPBAA levels are a direct representation of protein content in seeds.

Water limitation: in response to water limitation, TPBAA content per mg increased significantly (~23%) but only in response to the most severe water condition imposed, i.e., 10% PC (Figure 4a). TPBAA content per seed increased (11-60%) in plants maintained at 50% PC or less (Figure S5a), which is consistent with the alteration found in seed weight. Water deprivation: TPBAA content per mg fluctuated across the water deprivation treatments, but increased (13-23%) in plants that received 3 weeks or more of water deprivation stress (Figure 4c). TPBAA content per seed, however, decreased (16% and 21%) in response to the two most severe conditions imposed, i.e., 4WS and 5WS, respectively (Figure S5c). Interestingly, TPBAA content per seed correlated highly with seed weight in response to water limitation but only moderately in response to water deprivation (Table S3).

The relative composition of individual PBAAs did not change in response to either water stresses, despite the opposite outcomes in seed weight

To evaluate the effects that the various water stress treatments had on the relative composition of individual PBAAs, we calculated the percentage of each PBAA level to the TPBAAs measured in the seed (Figure 5a, b; Data S1e, f). Surprisingly, neither type of water stress nor severity had much of an effect on the relative composition of individual PBAAs (Figure 5a, b; Table S5).

Seed TFAA content per weight increased substantially only in response to severe water deprivation

Although most amino acids are protein bound, a small portion is actively maintained as FAA to serve in various biological processes. To evaluate how the various water stress treatments effected FAA levels, we used a targeted LC-MS/MS-MRM approach to quantify the proteogenic FAAs, excluding glycine, from dry seeds (Data S2a, b). The TFAA content per mg and per seed were also measured, and the results presented in Data S2.

Water limitation: In response to water limitation, TFAA content per mg increased only slightly (~12 %) and only in response to the most severe condition, i.e., 10% PC (Figure 4b). A more pronounced effect was observed in TFAA content per seed, which increased in plants receiving 25% PC and less by 16-45% (Figure S5b). The Spearman rank correlation between TFAA content per seed and seed weight showed a high positive correlation (Table S3a). Water deprivation: In response to water deprivation, the content of seed TFAA per mg was strongly elevated in plants exposed to 3 to 5 weeks of stress by 266-466% (Figure 4d). Similar trends were observed in the TFAA content per seed, 208-290% elevation in plants exposed to 3 weeks or more of water deprivation (Figure S5d). The Spearman rank correlation between TFAA content per seed and seed weight showed a negative correlation in seeds subjected to water deprivation (Table S3b).

The relative composition of individual FAAs in dry seeds shows major reprograming in response to water deprivation treatments, but not to water limitation treatments

To evaluate the effects that the various water stress treatments had on the relative composition of individual FAAs in dry seeds, we calculated the percentage of each FAA level to the TFAA measured (Figure 5; Data S2e, f). Water limitation: In response to water limitation, the relative composition of individual FAAs in dry seeds remained nearly unchanged for all conditions imposed. The only two amino acids that showed differences between the 100 vs 10% PC treatments were Glu (-6%) and Asp (~4%) (Figure 5c; Table S5c). Water deprivation: Dry seeds from plants subjected to water deprivation showed extensive alterations to the relative composition of many FAAs (Figure 5d; Table S5d). For example, Pro and Thr, which comprised only 2

and ~3%, respectively, of the TFAA pool in control seeds, were the most contributing compounds to the TFAA pool in seeds developed under 5 weeks of water deprivation stress, ~33 and 10%, respectively, (Table S5d).

Many strong positive correlations were found among and between seed FAA and PBAA in response to water limitation but not to water deprivation

To evaluate the relationships among the individual FAAs and PBAAs in response to the various water stress treatments, we performed a Spearman's rank correlation analysis from the individual seed FAA and PBAA per seed. The prefix "f" denotes free amino acids (e.g., fAla), and the prefix "b" denotes protein-bound amino acids (e.g., bAla).

Water limitation: In response to water limitation, we found almost exclusively positive correlations between all the detected amino acids. Only fGlu exhibited weak to no correlations with most of the amino acids, especially the FAA pool (Figure 6a). Interestingly, correlations within PBAAs were stronger compared to the correlations within FAAs or the correlations between the two pools (Figure 6a). Water deprivation: A very different outcome was observed in response to water deprivation. Despite the strong correlations within each amino acid pool, most correlations between the two pools were weak or largely insignificant (Figure 6b). We found strong to moderate negative correlations between specific PBAAs and a wide range of FAAs, especially for bLys, bPhe, and bArg. Interestingly, strong negative correlations between fTrp and almost all other FAAs were found. Moreover, fGlu showed no significant correlation with most amino acids from both pools. The data suggest a tight relationship between the FAA and PBAA pools in response to water limitation, but a very limited relationship between these pools in response to water deprivation.

The seed proteome underwent extensive proteomic reprogramming in response to severe water deprivation but not to severe water limitation

We evaluated the proteomic composition of dry seeds in response to the most severe water stress conditions (i.e., 10% PC and 4WS) and compared them with the controls (100% PC and 0WS) using a mass spectrometry-based identification of proteins. (The 5WS plants did not yield enough seed for this analysis.) The analysis identified 3538 proteins from the water limitation treatment, and 3648 proteins from the water deprivation treatment. Proteins with low spectral counts and poor reproducibility were removed, leaving 1506 (~46%) proteins from the water limitation treatment and 1690 (~46%) proteins from the water deprivation treatment (Data S3, S4). The significance of the proteome response to severe water stress was determined using a *t*-test followed by a False Discovery Rate (FDR) correction for multiple-comparisons to reduce the likelihood of

false positives. Stress-over-control ratios were statistically significant if the *p*-value after correction was less than 10%.

Water limitation: No protein was significantly altered in dry seeds of plants exposed to the most severe water limitation condition, i.e., 10% PC, which was consistent with the lack of change found in the relative composition of TPBAA under this same condition (Figure 5a). Water deprivation: In contrast, 576 proteins (~34% of the identified proteins) were significantly altered in seeds of plants exposed to 4 weeks of severe water deprivation, i.e., 4WS, (Data S5); although, the TPBAA composition of these seeds remained unchanged (Figure 5b). Of the altered proteins, 541 increased and 35 decreased in abundance (Data S5).

Seed proteins altered in response to severe water deprivation were enriched for stress-related processes, amino acid and protein metabolism, and nutrient reservoir activity

We used the Biological Network Gene Ontology (BiNGO) tool to investigate whether specific biological processes and molecular functions were overrepresented in the proteins altered in response to severe water deprivation, i.e., 4WS. We first performed a full GO term analysis of proteins that increased (Data S6a) and decreased (Data S6b). Notably, the proteins that increased in abundance included many terms related to osmotic stress, protein folding and catabolism, translation, and fatty acid degradation (Data S6). We next used GOSlim Plant ontology to better pinpoint the main biological processes and molecular functions enriched (Figure 7; Data S7). The proteins that increased were enriched for biological process terms related to response to stress and abiotic stimulus (Figure 7a1; Data S8); specific proteins included were the late embryogenesis abundant and heat shock proteins, glutathione-S-transferase, catalase, and ascorbate peroxidase (Data S8). Increased proteins were also enriched for carbohydrate metabolic process, photosynthesis, and generation of precursor metabolites and energy as well as both catabolic and biosynthetic processes (Figure 7a1); all these terms are biological processes known to be highly responsive to water stress conditions. Several terms associated with protein biosynthetic processes also were identified and included ribosomal proteins and translation initiation and elongation factors (Data S8). However, there were also terms related to protein degradation that included ubiquitin, proteasome, and several proteases. Multiple terms related to cellular amino acid metabolic process were also identified and were mostly biosynthetic and included proteins involved in Pro, Asn, Met, Trp, Thr, Arg, Lys, and Gly biosynthesis (Data S8). Very few enriched terms were identified in the molecular function category from the significantly increased proteins and included structural molecule and catalytic activity as well as translation factor activity, nucleic acid binding, and protein binding (Figure 7a2; Data S8).

Among the proteins that decreased in dry seeds in response to severe water deprivation, no significant biological process terms were enriched, and only molecular function terms were enriched under the full GO ontology ($p < 0.05$) (Data S6b). The most significantly enriched term in this group was nutrient reservoir activity, which included the three most abundant SSPs in *Arabidopsis* (cruciferin 3, cruciferin 2, and cruciferin 1) and other storage proteins, including the RmlC-like cupins superfamily proteins and seed storage albumin 3 (Figure 7b; Data S8).

DISCUSSION

Plants are routinely exposed to sporadic or extended episodes of drought or limited amounts of rainfall. Understanding how these conditions affect seed amino acid levels and composition is important if breeding strategies aimed at improving seed nutritional and agronomical qualities under water-stressed conditions are to be considered. However, little is known about the effects that various water stress conditions, especially those imposed during the seed setting stage, have on the content or relative composition of the two amino acid functional pools (FAAs and PBAAs) in the dry seeds. To bridge this informational gap, we conducted a comprehensive analysis of seed physiological and metabolic responses to a wide range of water stress intensities and impositions. The outcomes at both sample and whole-seed levels and the potential molecular mechanisms that underlie these outcomes are summarized in Figure 8.

Plants employ different acclimation strategies in response to severe water limitation and severe water deprivation, which results in fewer and bigger seeds in the former and fewer and smaller seeds in the latter

To understand the physiological context of FAA and PBAA responses, we first assessed the impact of the two stress treatments on key physiological parameters: leaf RWC, yield, and weight. Our data indicate that in response to both types of water stress, plants attempted to maintain their leaf RWC for as long as possible after the decline in soil RWC. It was previously shown that plants exposed to drought can maintain their leaf RWC for a certain period of time, depending on the species, before dropping sharply thereafter. Resurrection plants, for example, can maintain their leaf RWC for more than 10 days after drought imposition (Oliver *et al.*, 2011). In our study, most plants subjected to a water limitation stress were able to maintain a normal leaf RWC (~88%) throughout the duration of the experiment; the only exception were those plants exposed to the most severe treatment (i.e., 10% PC), which was reduced to ~50% three weeks after the stress was imposed (Figure

S1). These findings show that the plants can acclimate to reduced but sustained amounts of water supply. Under water deprivation, however, the plants maintained a leaf RWC at control levels only for a week after a drop in soil RWC (Figure S2), which suggests that this type of water stress was more acute and challenging to overcome. These observation were consistent with the differences found between the two water stress impositions in both seed yield and weight outcomes. Although in both treatments seed yield was considerably reduced, plants exposed to 4 or 5 weeks of water deprivation displayed a more pronounced reduction than the most severe water limitation, i.e., 10% PC (Figure 2). In addition, seed weight decreased by up to 31% in response to water deprivation but increased by up to 30% in response to water limitation (Figure 2). Alteration in seed weight can be traced to changes in seed filling rate as well as to the production of seed storage reserves, such as starch, oil, or proteins (Barnabas *et al.*, 2008, Stagnari *et al.*, 2016, Sehgal *et al.*, 2018), which are dependent on the amount of nitrogen and carbon supplied to seeds. For instance, water stress inhibits cell division in endosperm cells and the number of starch granules in grains restricting seed size in cereals (Arakawa and Timasheff, 1985, Fang *et al.*, 2010, French and Turner, 1991, Brocard *et al.*, 2017, Sehgal *et al.*, 2018). Both elevation and reduction in seed weight have been observed previously. For example, a study of soybean seeds reported an increase in size as water limitation increases (Adjei-Twum and Splittstoesser, 1976), while other studies have reported that water stress led to reductions in seed weight in soybean, cereals, legumes, and *Brassicas* (Champolivier and Merrien, 1996, De Souza *et al.*, 1997, Brevedan and Egli, 2003, Barnabas *et al.*, 2008, Farooq *et al.*, 2017, Hatzig *et al.*, 2019, Sharma *et al.*, 2018, Sehgal *et al.*, 2019). Our data suggest that such discrepancies may arise from the type of water stress imposed or the plant's ability to acclimate to it. It has been previously hypothesized that plants exposed to stress reduce their seed number to conserve resources and allocate them to fewer vital seeds rather than more seeds (Lloyd, 1980, Sehgal *et al.*, 2018). As shown in Figure 8, our findings support this hypothesis and demonstrate that seed number and weight are not predetermined upon bolting and can be altered in response to stress at almost any stage during seed setting (Figure 2) and, moreover, that plants can independently regulate both depending on the type and severity of water stress experienced and their capacity to acclimate to it.

Severe water limitation and deprivation led to similar metabolic adjustments of carbon, nitrogen, and PBAA per weight despite opposite trends at the whole-seed level

To further understand the metabolic outcomes in dry seeds in response to water stress, we evaluated carbon, nitrogen, TPBAA, and total lipids, which represents the main storage compound in *Arabidopsis* seed (Baud *et al.*, 2002, Li *et al.*, 2006, Baud *et al.*, 2008). We measured the latter at the whole-seed level as well as per weight since our experiments showed that seed weight was altered in response to the type and severity of the

water stress imposed. In response to water limitation, both nitrogen and carbon content per seed increased in seeds exposed to 50% PC or less, which was consistent with the elevation in proteins (TPBAA), lipids, and seed weight (Figures S4, S5; Table S4). These observations support the hypothesis that, in response to these conditions, the plant allocates more resources to each seed. In contrast, nitrogen, carbon, lipid, and protein (TPBAA) content per seed were reduced in response to water deprivation (Figures S4, S5; Table S4). Reduction in oil content in response to water stress has been reported previously in soybean, maize, and *Brassica napus* (Dornbos *et al.*, 1989, Sinaki J. M., 2007, Bilibio et *al.*, 2011, Ali *et al.*, 2012). Nevertheless, the protein content response to water stress is less clear: some studies report decreases in protein content (Bouchereau *et al.*, 1996, Ghanbari, 2013, Sehgal *et al.*, 2019), while others report increases in protein content (Bouchereau *et al.*, 1996, Behboudian *et al.*, 2001, Gooding *et al.*, 2003, Teixeira and Pereira, 2007) . Our results suggest that these observations may emanate from different water stress severity and imposition. However, our findings also show that nitrogen levels per seed increased more than carbon in response to severe water limitation, i.e.,10% PC (~44% versus 36%) and decreased less than carbon in response to severe water deprivation, i.e., 4WS, (22% versus ~33%). These metabolic trends led to surprisingly similar metabolic outcomes when comparing equal seed weight, showing that in response to severe stress, regardless of type, the carbon relative content (per mg) was unaltered while nitrogen content increased. These observations agree with findings in cereals that have shown a decrease in the carbon/nitrogen ratio in response to water stress as a result of more nitrogen assimilation in seed (Sehgal *et al.*, 2018). The TPBAA content per mg also increased regardless of water stress imposition or severity. However, lipid content per mg was reduced substantially, while carbon content per mg was unaltered in response to water deprivation, suggesting that other carbohydrates increased. Indeed, previous water stress studies have reported that seeds accumulate carbohydrates such as sucrose, galactinol, and raffinose to protect them from oxidative damage (Taji *et al.*, 2002,Nishizawa-Yokoi *et al.*, 2008). Taken together, our results show that, in response to a wide range of water stress conditions and despite opposite metabolic trends at the whole seed level, the carbon relative content is maintained while the nitrogen relative content increased when comparing equal sample weights. The first is achieved by alteration of lipids or other compounds, most likely sugars, and the second by alteration of the relative protein content (TPBAA per mg) (Figure 8).

PBAA composition is rigorously maintained in response to severe water limitation and deprivation but by two distinct molecular mechanisms, one of which is proteomic rebalancing

In addition to the common responses described above, we also found that the overall PBAA composition remained unaltered even under the most severe water stress conditions (Figure 4a, c). This finding aligns well

with the preservation of the proteomic composition under the most severe water limitation treatment (10% PC) and suggests that the seeds did not undergo major compositional alterations despite an increase in weight and in TPBAA content per seed (Figures 2b, 5Sa). The maintenance of overall PBAA composition in response to severe water deprivation, however, was puzzling since over a third of the quantifiable proteome was significantly altered (Data S5). Approximately 93% of the altered proteins increased. GO Slim enrichment analysis of these proteins revealed enrichment for terms related to abiotic and biotitic stress, carbohydrate, amino acids, energy, and protein metabolism as well as terms related to embryo development (Figure 7; Data S8). A more detailed enrichment analysis (Full GO) further revealed terms related to osmotic stress, protein folding and catabolism, translation as well as fatty acid degradation (Data S6). These findings indicate that the proteome has undergone proteomic reprogramming aimed at ensuring osmoregulation and protection through metabolic adjustment. Elevation of specific proteins, such as heat shock and late embryogenesis abundant proteins as well as antioxidant enzymes further support this claim (Data S8). Enrichment of similar processes has been reported in the dehydrating leaves of the resurrection plant *Sporobolus stapfianus* (Yobi *et al.*, 2017 and references therein). As with resurrection plants, developing seeds exposed to severe water deprivation undergo proteomic reprogramming that consists of an increase in the abundance of osmolytic and defense related proteins even though they are inherently programmed for desiccation-tolerance. The proteins that decreased in response to severe water deprivation were enriched for nutrient reservoir activity proteins, including the most highly abundant SSPs in *Arabidopsis*—CRU1, 2, and 3 (Data S6, S8). Alteration of storage proteins in response to water stress has been reported in peas, soybean, wheat, and lentil (Ghanbari et *al.*, 2013, Zhang *et al.*, 2014, Nakagawa *et al.*, 2018, Sehgal *et al.*, 2019).

Taken together, our overall analysis revealed that the majority of the increased proteins are non SSPs, while the decreased ones are enriched with SSPs; yet, no alteration was observed to the overall PBAA composition. The SSPs have a distinct amino acid composition characterized by low levels of specific essential amino acids (Li *et al.*, 2018, Ren *et al.*, 2018) and, therefore, are known to negatively affect a seed's nutritional quality (Herman and Larkins, 1999, Don *et al.*, 2006, Nguyen *et al.*, 2015). In theory, the increase in non SSPs and decrease in SSPs should have resulted in the alteration of the composition of PBAAs and alteration of the seed's nutritional quality. But, maintenance of the PBAA despite major alterations to the proteome revealed that a major proteomic rebalancing occurred. This phenomenon has been previously reported in SSP mutants of soybean, corn, barley, and *Arabidopsis*, indicating that it is a highly conserved mechanism (Geetha *et al.*, 1991, Kinney *et al.*, 2001, Hunter *et al.*, 2002, Withana-Gamage *et al.*, 2013). Studies of the maize opaque endosperm mutants, which exhibits a large reduction in SSPs, suggest that proteomic rebalancing may be facilitated by changes to only a few specific proteins (Schmidt *et al.*, 2011, Morton *et al.*, 2016). Proteomic

rebalancing under other conditions has not been reported, and its natural function remains unknown. Here, we show that seeds exposed to severe water stress undergo proteomic reprogramming as well as proteomic rebalancing. Moreover, we hypothesize this to be the original purpose of the mechanism – that is, to facilitate the maintenance of a seed's relative PBAA composition in response to changing environmental conditions (rather than to counteract severe mutations in highly abundant proteins, such as SSPs). This hypothesis is supported by the finding that the PBAA composition of seeds was tightly regulated across all water stress conditions, which also strongly indicates that PBAA composition is essential to seed development and survival.

FAA composition is altered in response to severe water deprivation but not water limitation, indicating plasticity in the FAA response

Despite an increase in seed total FAA in response to severe water limitation (10%PC) (Figure 4b, S5b), no alterations were observed in seed FAA composition (Table S5c). In contrast, major seed FAA reprogramming occurred in response to severe water deprivation (Figure 4d). This reprogramming was characterized by a notable increase in amino acids, including Proline, which is important in osmotic adjustment and water stress tolerance (Hayat *et al.*, 2012, Dar *et al.*, 2016). An increase in and a reprogramming of FAAs in the vegetative tissues in response to water stress have been reported in *Arabidopsis*, maize, cotton, tomato, and *S. stapfianus* (Ranieri *et al.*, 1989, Pérez-Alfocea *et al.*, 1993, Showler, 2002, Martinelli *et al.*, 2007, Oliver *et al.*, 2011, Batista-Silva *et al.*, 2019) . It has been proposed that this response may mitigate the negative effects of the stress and may emanate from either inhibition of protein synthesis, protein turnover, or altered balance between biosynthesis and catabolism of amino acids (Martinelli *et al.*, 2007, Barros *et al.*, 2017, Huang and Jander, 2017, Hildebrandt, 2018, Hirota *et al.*, 2018). Our proteomic data showed an increase in several amino acid biosynthetic proteins (Data S8) as well as in terms related to protein biosynthesis and degradation (e.g., ribosomal proteins, 26S proteasome), which suggests that the increase in seed FAA levels may have originated from both protein turnover and amino acid synthesis. However, we cannot rule out increased amino acid transport from vegetative tissues. The different FAA responses between the treatments (Figure 8) further supports that seeds underwent osmotic adjustment only under severe water deprivation and also indicate that FAA response to water stress is more plastic that the PBAA response. This finding is consistent with multiple observations of the responsive and adaptable nature of FAA traits in plants (Martinelli *et al.*, 2007, Oliver *et al.*, 2011, Lanzinger *et al.*, 2015, Yobi *et al.*, 2019).

FAA and PBAA respond independently to severe water deprivation despite their close metabolic relationship

This study sheds new light on the complex relationship between the FAA and PBAA functional pools in dry seeds. FAAs integrate into proteins during seed development and then convert to PBAAs. Since they are also precursors for many other metabolites, their steady state levels may be driven, at least in part, by general cell metabolism (Fait *et al.*, 2006, Gutierrez *et al.*, 2007, Frank *et al.*, 2015, Tan *et al.*, 2015). In our experiments, plants exposed to a water limitation stress during their seed setting stage yielded heavier seeds with elevated levels of both FAA and PBAA pools, phenotypes that were significantly correlated (Figure 6a), suggesting some degree of interdependency. However, plants subjected to a water deprivation stress had no correlation between the two amino acid functional pools (Figure 6b). Rather, the FAA composition underwent reprogramming most probably due to osmotic adjustment, while the PBAA composition remained unaltered due to proteomic rebalancing, indicating that distinct regulatory mechanisms govern these two functional pools.

CONCLUSION

We found that plants use different strategies in response to varying types and degrees of water stress encountered during the seed setting stage. These strategies result in different outcomes at the whole-seed level, but several similar ones when comparing equal seed weights (summarized in Figure 8). However, more importantly, this study revealed that the PBAA relative composition is rigorously maintained, strongly suggesting that PBAA composition plays a key role in seed survival under any water stress condition. Furthermore, PBAA maintenance is facilitated by two distinct molecular mechanisms: proteomic maintenance in response to water limitation and proteomic rebalancing in response to water deprivation. The latter mechanism suggests a natural function for this phenomenon. In contrast to PBAA, we show that the FAA relative composition depends on the type and severity of the water stress, further supporting the adaptable nature of FAA in plants and also revealing a distinct response of the two amino acid functional pools in seeds despite their close metabolic relationship.

EEPERIMENTAL PROCEDURES

Plant growth and seed collection

Arabidopsis plants were grown in 3.5-inch pots in a 16-h light/8-h dark cycle and at a constant temperature (21-22ºC). Plants were grown under well-watered conditions until the onset of bolting. At this stage, plants that showed comparable initial bolting stages were selected for a water limitation or a water deprivation regimen.

Water stress treatments

The water limitation experiment consisted of daily monitoring of pot capacity (PC) and adequate watering to sustain 10, 25, 50, 75, or 100% PC. For each target treatment, 5 pots ($n = 5$) each containing two plants were used. The experiment was initiated one week after the bolting stage. The 100% PC (control) was maintained at saturation by daily monitoring and adding water as needed. Plants grown under the other treatments were allowed to slowly dehydrate until their respective target PCs were reached and were then maintained the same way as in the 100% PC.

For the water deprivation experiment, plants were subjected to one of five different durations of water withholding treatments, starting one week after initial bolting. For each treatment, 5 pots ($n = 5$) each containing two plants were used. Those plants that received 5 weeks of stress (WS) after bolting were the most stressed treatment. The 4W, 3W, 2W, and 1W plants were stressed for 4, 3, 2, and 1 week, respectively. Since the growth period ended around six weeks under our conditions, the plants that received six weeks of irrigation (i.e., 0WS) served as controls.

Soil and leaf water content measurements

Soil water content (WC) was measured using a soil sensor reader (Spectrum Technologies, Inc.). These measurements were converted to relative water content (RWC) as detailed in Methods S1a. Leaf RWC was measured from three pots ($n = 3$) for each treatment. From each pot, three leaves were removed once a week and weighed to determine the fresh weight (FW). The leaves were placed in deionized water overnight at 4ºC (in the dark) to measure the turgor weight (TW) and then dried in an oven at 70ºC for 48 h to measure the dry weight (DW). The formula (FW-DW)/(TW-DW) *100 was used to determine the RWC for each treatment.

Seed yield and seed weight estimates

Seeds were harvested throughout the seed setting period from all treatments ($n = 5$) to prevent seed loss. The final seed yield was determined by combining all the harvests from a single pot. Seed weight was measured by weighing ~1000 seeds in triplicates ($n = 3$) from all treatments and then dividing weight by seed count.

Seed moisture content and germination rate

To obtain the dry weight, seed moisture content was measured as described in (Baud *et al.*, 2002) by weighing ~10 mg of seed from three biological replicates of each treatment ($n = 3$) and drying them at 50ºC for 48h. Seed moisture was calculated using the formula 100*(W1-W2)/W2, where W1 is the weight before drying and W2 is the weight after drying. For the germination rate, four plates each containing 50 seeds per treatment were

sterilized with 10% bleach and then spread on agar plates (8g/L) with no MS added. Plates were sealed with 3M Micropore Paper Tape, incubated overnight at 4ºC, and then incubated at room temperature under constant light. Three days later, germination rate was determined by counting seeds that displayed radicle protrusions.

Amino acid analysis

For all treatments, five biological replicates were used for analyzing free and protein bound amino acids. The analyses were performed using an ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) instrument (Waters Corporation, Milford, MA) as described in detail in Methods S1b. PBAAs were analyzed by acid hydrolysis followed by extraction using ~3 mg tissue from the five biological replicates ($n = 5$) as described in (Yobi and Angelovici, 2018). The FAA analyses were extracted from ~6 mg tissue from 5 biological replicates of each treatment ($n = 5$) as described in (Yobi *et al.*, 2019).

Nitrogen and carbon analyses

Nitrogen and carbon were determined from ~2 mg tissue from 3 biological replicates of each treatment ($n = 3$) by combustion and gas chromatography using an ECS 4010 CHNSO analyzer (Costech Analytical Technologies, Inc., Valencia, CA) as detailed in Methods S1c.

Lipid analysis

Lipids were analyzed based on (Folch, 1957) as described by (Li *et al.*, 2006, Brocard *et al.*, 2017). Briefly, ~10 mg seeds harvested from plants subjected to the 10% PC and 4WS treatments and their respective controls ($n = 3$) were used for total lipid extraction. The analysis was performed using a gas chromatography with a flame ionization detector, as described in detail in Methods S1d.

Proteomic analysis

Protein extraction was performed as described in (Hurkman and Tanaka, 1986). Briefly, 5 mg seeds were weighed and extracted using phenol and SDS extraction followed by trypsin digestion and analysis with a Bruker timsTOF Pro MS/MS (trapped ion mobility spectrometry time-of-flight spectroscopy) platform, as described in detail in Methods S1e.

Data analysis

To determine if there were coordinated responses (a) among amino acid, seed weight, carbon, and nitrogen levels and (b) within and between the two amino acid pools, we performed a pairwise Spearman's rank correlation analysis using the same platform as previously described (Batushansky *et al.*, 2016). We visualized

the correlation matrix in R v.3.4.3 (R Core Team, 2014) using the corrplot package (Wei and Simko, 2017). The significance of the correlation coefficients (*r*-values) was based on the corresponding q_{FDR}-*values* < 0.05. |r|-values < 0.45 had q_{FDR}-values > 0.05, so were considered not significant. Only correlations with |r|-values > 0.41 were deemed significant.

For the proteomic analysis, the significance of the stressed versus control treatments was determined by *t*-test after a 10% FDR correction.

Enrichment analysis was performed using the Cytoscape plug-in BiNGO v3.0.3 (Maere *et al.*, 2005). The following parameters were used to determine the gene ontology (GO) biological process and molecular function terms that were overrepresented ($p < 0.05$): a hypergeometric test with a 5% *FDR* correction ($n = 3$), a custom reference that consists of the proteins that were detected in our study, *Arabidopsis* as the select organism, and both GO Full and GOSlim Plant ontologies.

Data availability statement

All amino acid and proteomics raw data are available in the supplementary datasets and their analyses are in the supplemental tables. Seed relative water content and germination data as well as total lipid measurements are available in the supplementary data associated with this manuscript.

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AUTHOR CONTRIBUTIONS

AY supervised the experiments and wrote the manuscript. CB performed the experiments. TM performed lipid analysis. SH and NM performed phenotypical measurements. AB, VS, ST-H, and ME performed statistical data analyses. RA designed, analyzed and supervised the experiments and wrote the manuscript. All authors have reviewed the final version of the manuscript and approved it and therefore are equally responsible for the integrity and accuracy of its content.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

SUPPORTING INFORMATION LEGENDS

Additional supporting information may be found in the online version of this article:

Figure S1. Soil and leaf relative water content of *Arabidopsis* plants subjected to water stress.

Figure S2. Soil and leaf relative water content of *Arabidopsis* plants subjected to water stress.

Figure S3. Representative images of *Arabidopsis* plants after water stress.

Figure S4. Nitrogen and carbon absolute levels (µg/seed) of seeds from water-stressed *Arabidopsis* plants.

Figure S5. Protein-bound and free amino acids absolute levels (nmol/seed) of seeds from water-stressed *Arabidopsis* plants.

Data S1. Summary of the raw and normalized PBAA of seed from water-stressed *Arabidopsis* plants.

Data S2. Summary of the raw and normalized FAA in seeds subjected to water stress

Data S3. List of proteins detected in seeds in response to severe water limitation.

Data S4. List of proteins detected in seeds in response to severe water deprivation.

Data S5. List of proteins that increased or decreased significantly in seeds in response to severe water deprivation.

Data S6. Full GO enrichment of proteins that increased or decreased significantly in seeds in response to severe water deprivation.

Data S7. GO Slim Plant enrichment of proteins that increased significantly in seeds in response to severe water deprivation.

Data S8. Representative proteins enriched among proteins that increased or decreased significantly in response to severe water deprivation.

Table S1. Relative water content of seeds from water-stressed *Arabidopsis* plants.

Table S2. Germination rates of seeds from water-stressed *Arabidopsis* plants.

Table S3. Spearman rank correlation between seed weight, total free amino acid (TFAA), total protein-bound amino acid (TPBAA), carbon, and nitrogen levels of seeds from water-stressed *Arabidopsis* plants.

Table S4. Total lipid composition of seeds from water-stressed *Arabidopsis* plants.

Table S5. Protein-bound and free amino acid compositions of seeds from water-stressed *Arabidopsis* plants.

Methods S1. Supporting experimental procedures.

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FIGURE LEGENDS

Figure 1. Two water impositions applied to *Arabidopsis* plants during the seed setting stage. The treatments consist of 100, 75, 50, 25, and 10% pot capacity (PC) watering regimes for the water limitation experiment (a) and of 0, 1, 2, 3, 4, or 5-week water stress (WS) regimes for the water deprivation experiment (b). Five plants were used for each treatment (n =5). All treatments were initiated one week after initial bolting and were applied until the completion of the seed setting period, which occurred six weeks after bolting under our growth conditions.

Figure 2. The effects of increasing intensities and durations of water stress imposed during the seed setting stage on seed yield and weight. The water limitation and deprivation treatments are described in Figure 1. (a) and (c) summarize the percentage of seed yield per plant relative to the control (n =5) under water limitation and deprivation treatments, respectively. (b) and (d) summarize the percentage of seed weight relative to the control (n =3 plants x ~1000 seeds) under water limitation and deprivation treatments, respectively. Error bars represent standard error. Duncan's Multiple Range Test was used to compare seed yield and weight among the treatments, with different lower-case letters indicating significant differences at the 5% level. Numbers in parentheses represent the percentage of increase or decrease compared to control levels.

Figure 3. The effect of increasing intensities and durations of water stress imposed during seed setting on nitrogen and carbon content per mg in *Arabidopsis* dry seeds. The water limitation and deprivation treatments are described in Figure 1. Nitrogen and carbon content per mg presented in (a) and (b) are measured from seeds exposed to water limitation conditions, while (c) and (d) present their content under water deprivation conditions. Error bars represent standard error (n = 3). Duncan's Multiple Range Test was used to compare nitrogen and carbon relative abundances among the treatments, with different lower-case letters indicating significant differences at the 5% level.

Figure 4. The effects of increasing intensities and durations of water stress imposed during the seed setting stage on TPBAA and TFAA content per mg in *Arabidopsis* dry seeds. The water limitation and deprivation treatments are described in Figure 1. The average TPBAA and TFAA content per mg under water limitation treatments are summarized in (a) and (b), respectively. The average TPBAA and TFAA content per mg under water deprivation treatments are summarized in (c) and (d), respectively. Error bars represent standard error (n =5). Duncan's Multiple Range Test was used to compare TPBAA and TFAA content among the treatments, with different lower-case letters indicating significant differences at the 5% level. Numbers in parentheses represent the percentage of increase or decrease compared to control levels.

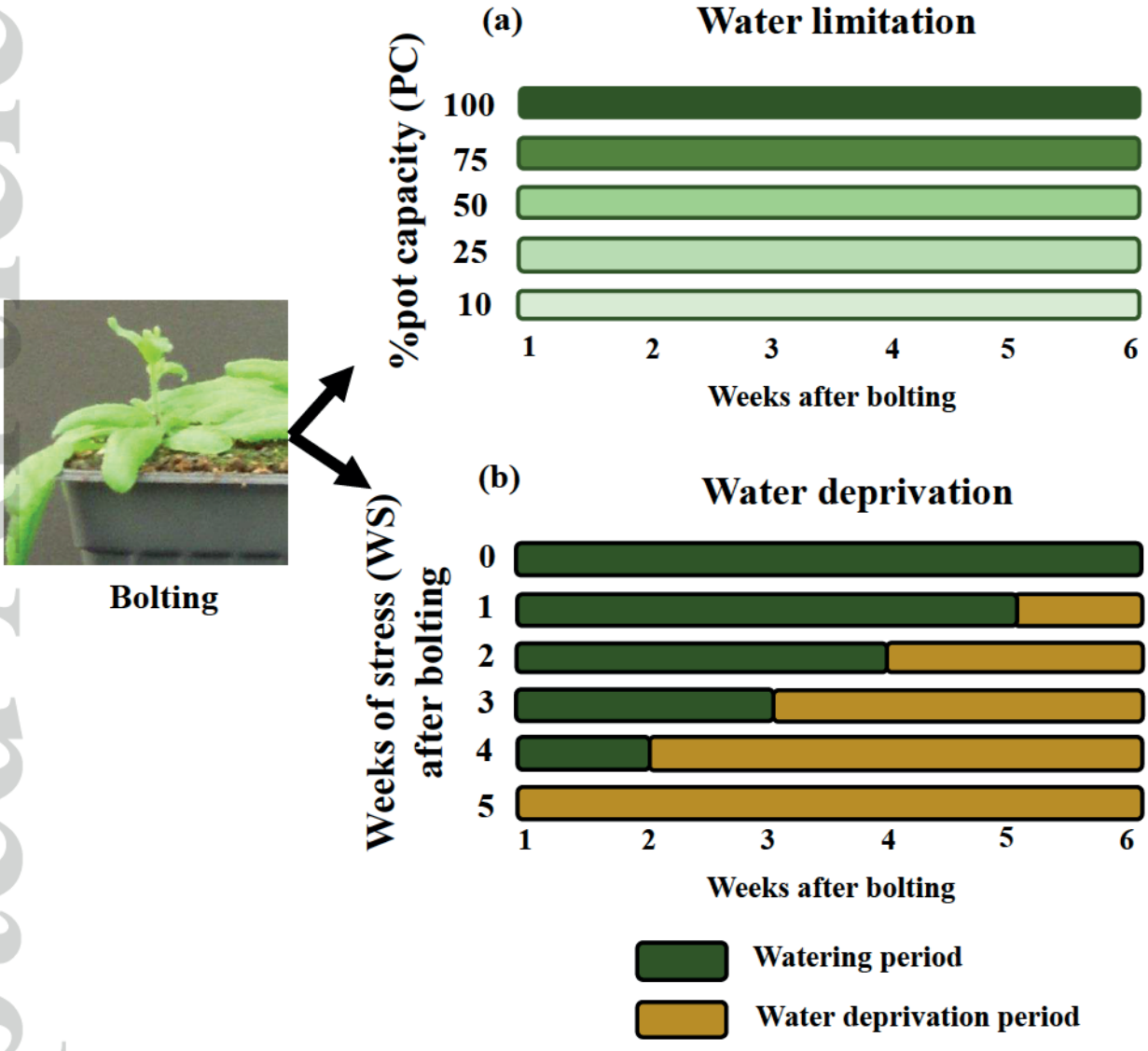
Figure 5. The effects of increasing intensities and durations of water stress imposed during seed setting on the relative composition of individual protein bound amino acids (PBAAs) in *Arabidopsis* dry seeds. The water limitation and deprivation treatments are described in Figure 1. (a) and (b) show the PBAA relative levels (%PBAA/TPBAA) in seeds harvested from plants exposed to water limitation and deprivation treatments, respectively. (c) and (d) show the FAA relative levels (%FAA/TFAA) in seeds harvested from plants exposed to water limitation and deprivation treatments, respectively. Error bars represent standard errors (n =5). The statistical significance of the ratios was determined by t-test, and the asterisk (*) indicates significance at the 5% level.

Figure 6. Spearman rank correlation analysis between seed FAA and PBAA content per seed from plants subjected to increasing intensities and durations of water stress imposed during the seed setting stage as described in Figure 1. (a) and (b) show the correlation heatmaps under water limitation and deprivation treatments, respectively. The correlation matrix was visualized in R v.3.4.3 (R Core Team, 2014). Each dot represents a significant correlation coefficient (r-values q_{FDR}-$values < 0.05$). Darker blue dots indicate higher positive correlations, darker red dots indicate higher negative correlations, and blanks indicate no significant

correlations. The prefixes "f" and "b" before the amino acids (e.g., fAla and bAla) denote free and protein-bound amino acids, respectively.

Figure 7. Gene ontology (GO) terms associated with proteins that increased or decreased in dry seeds of *Arabidopsis* plants subjected to a severe water deprivation treatment as described in Figure 1. Biological Network Gene Ontology (*BiNGO*) was used for the enrichment analysis. (a1) and (a2) show the biological process and molecular function terms enriched ($p < 0.05$) in the proteins that increased. (b) shows the molecular function terms enriched in the proteins that decreased. Plant GO Slim was used for (a1) and (a2), while Full GO was used for (b). Node size represents the number of genes within the node, and the color represents the *p*-value ($p < 0.05$) with darker orange indicating higher significance and no shading indicating no significance.

Figure 8. The physiological and metabolic outcomes in *Arabidopsis* dry seeds in response to severe water limitation and severe water deprivation. (a) summarizes the physiological (a1) and metabolic (a2) responses under severe water limitation treatment. (b) summarizes the physiological (b1) and metabolic (b2) responses under severe water deprivation treatment. FAA; Free amino acids, TPBAA; total protein-bound amino acids.



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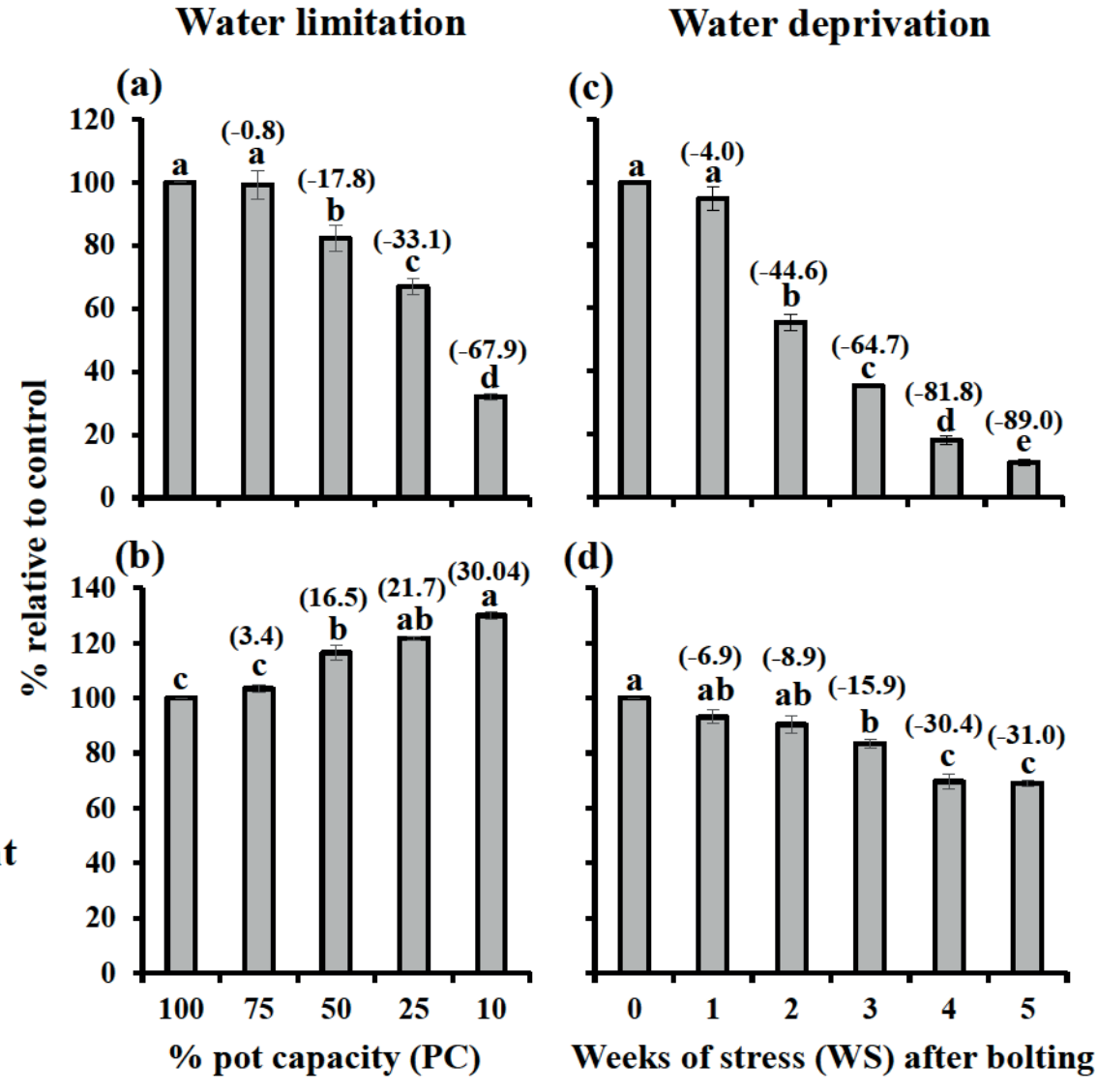

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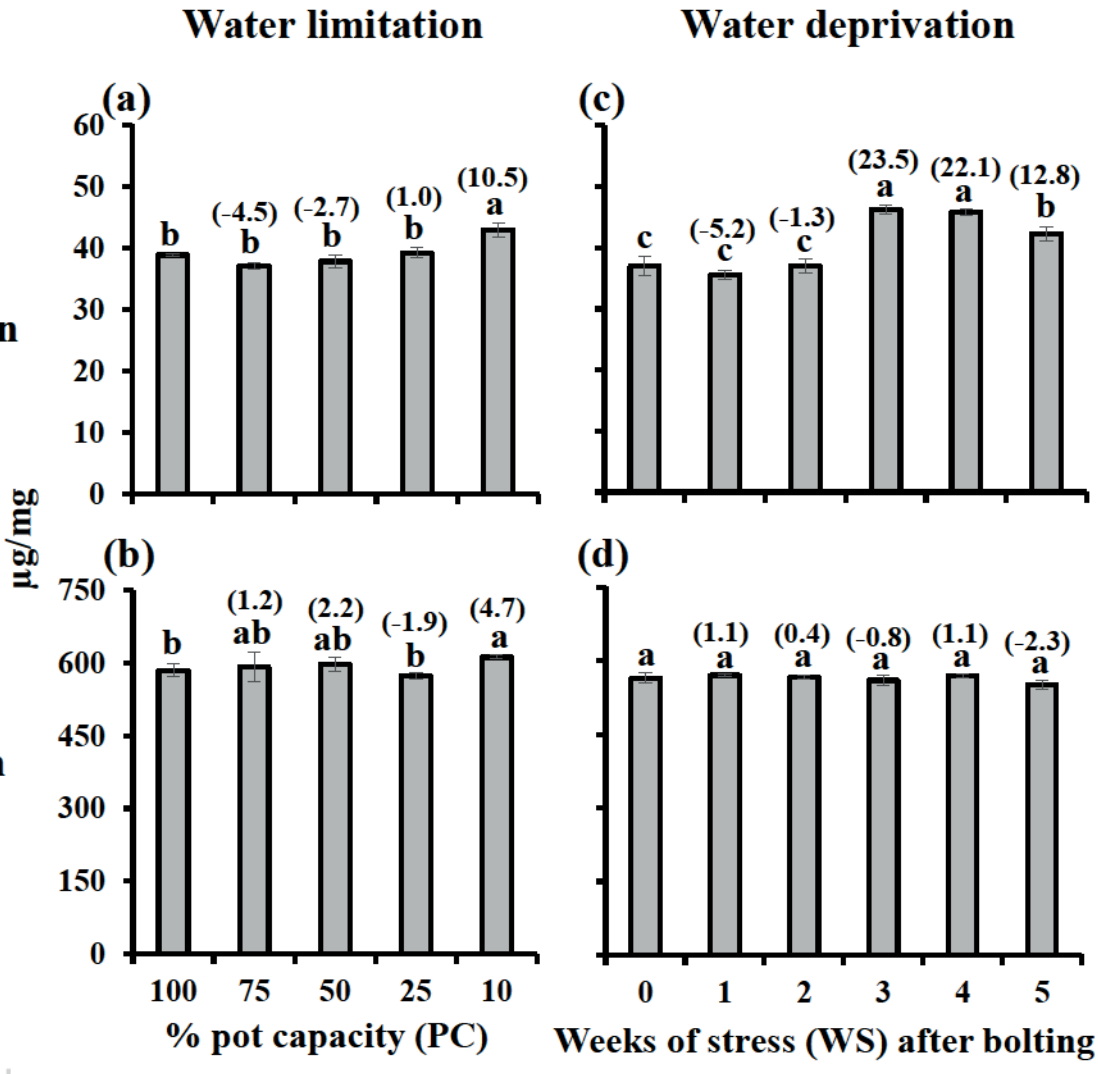

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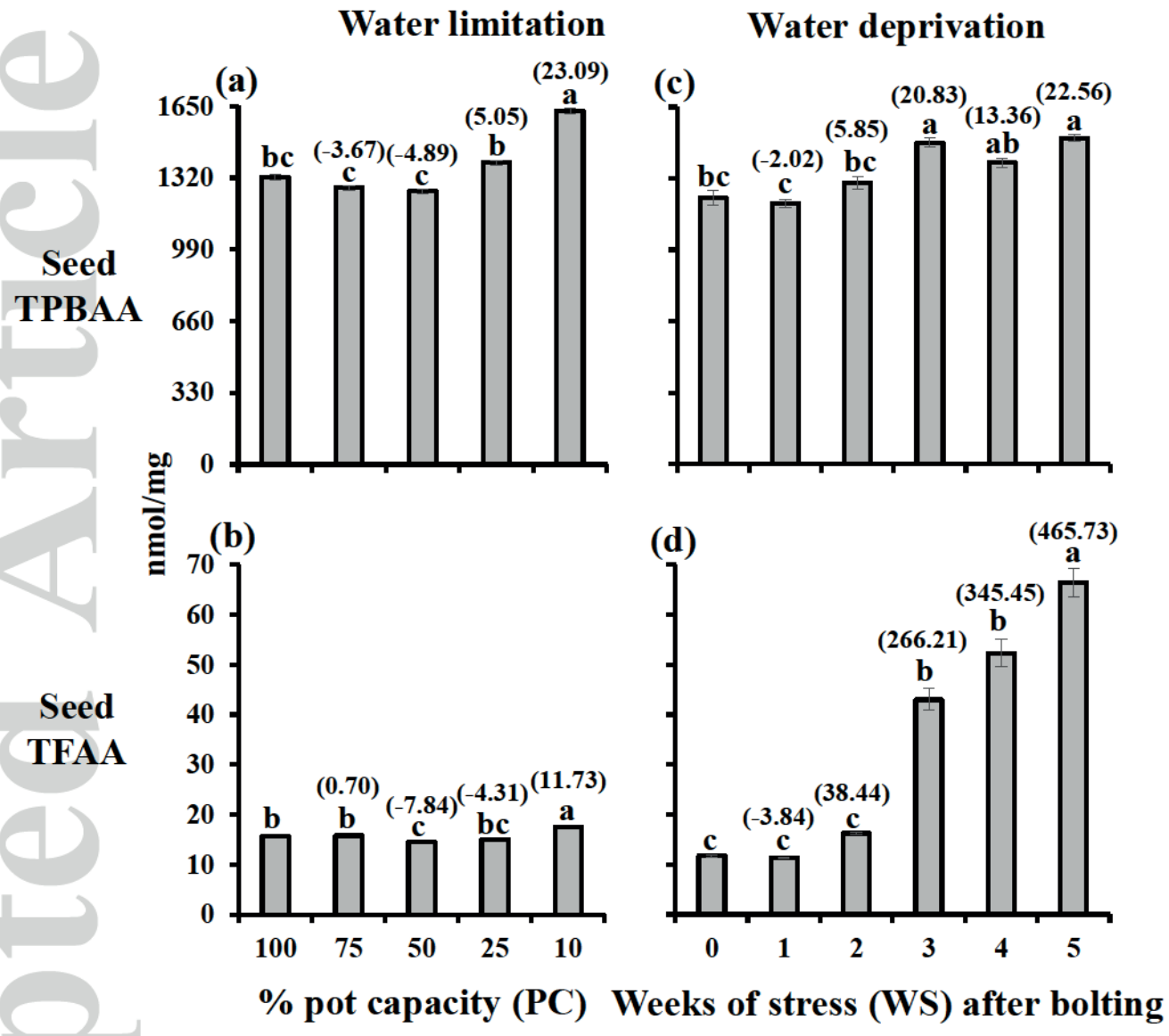

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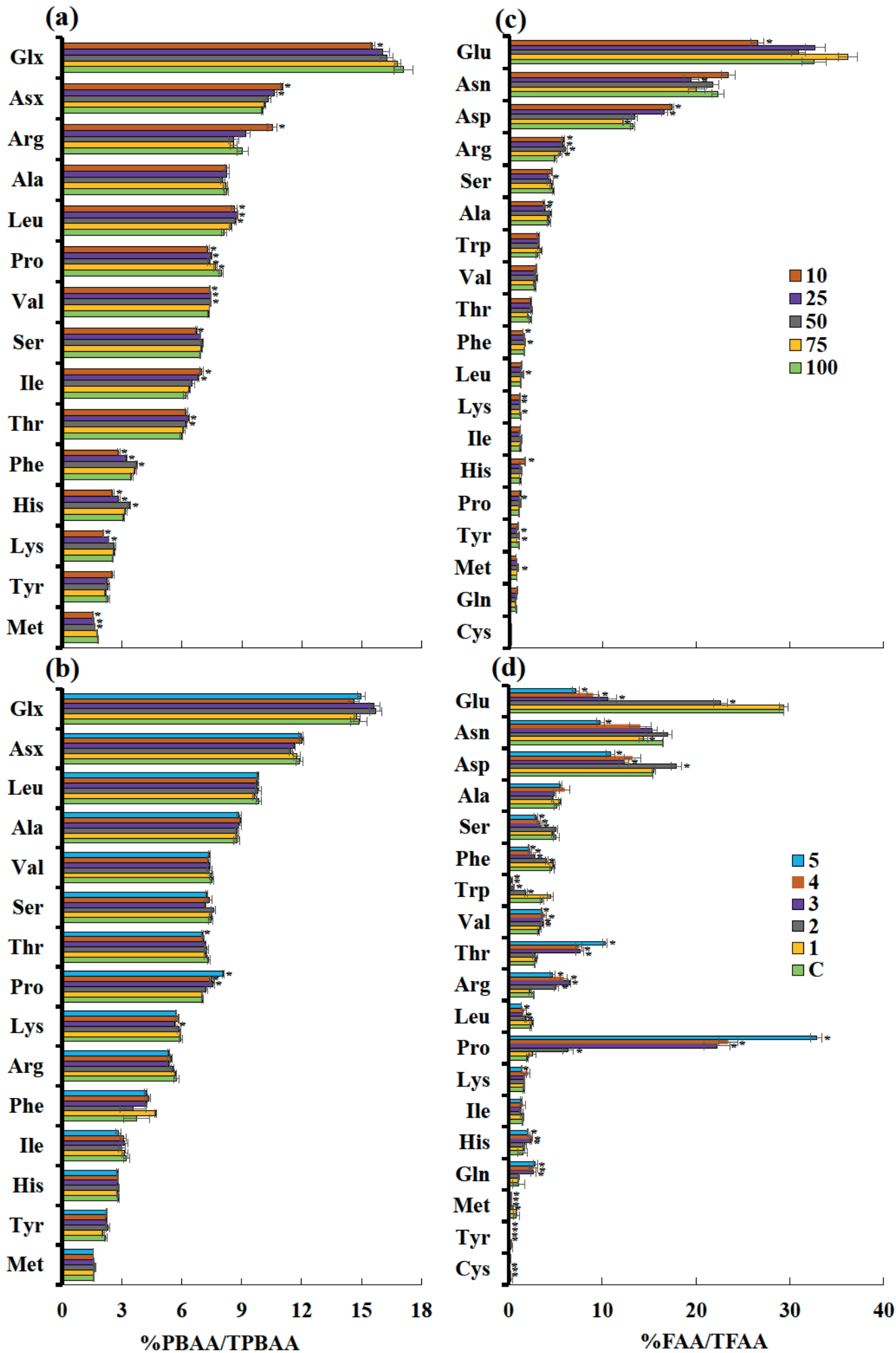
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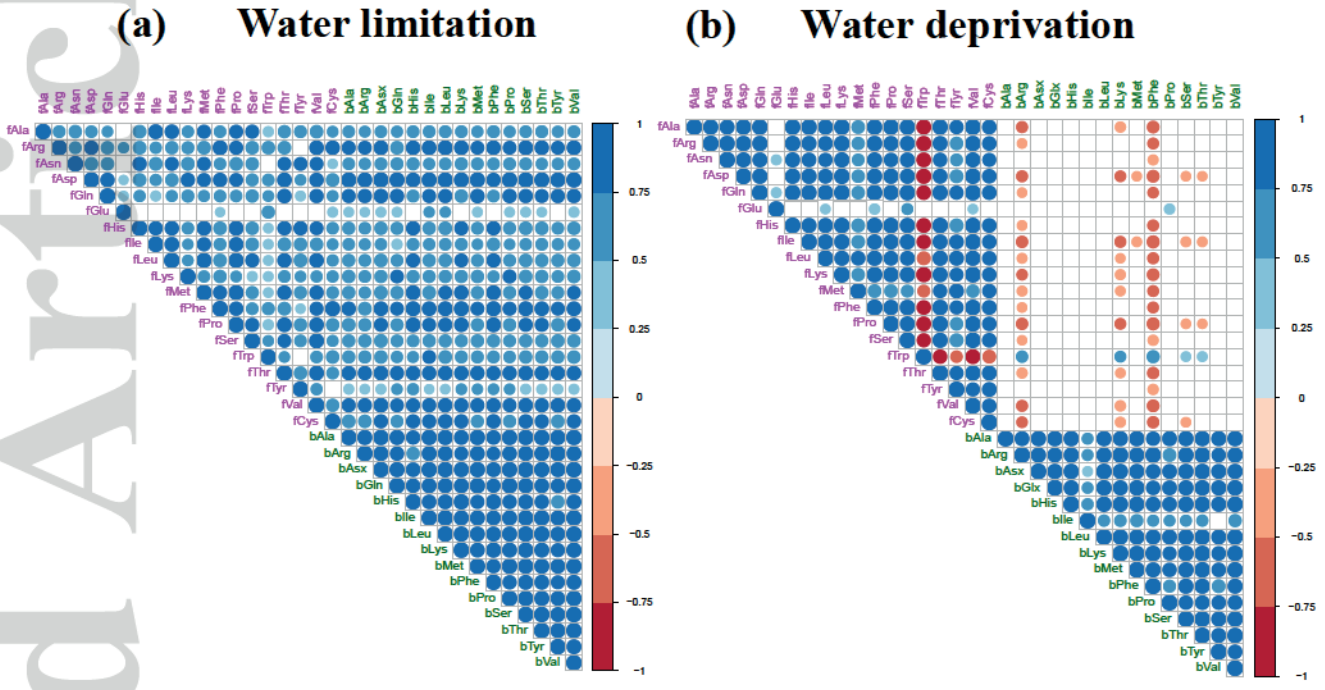

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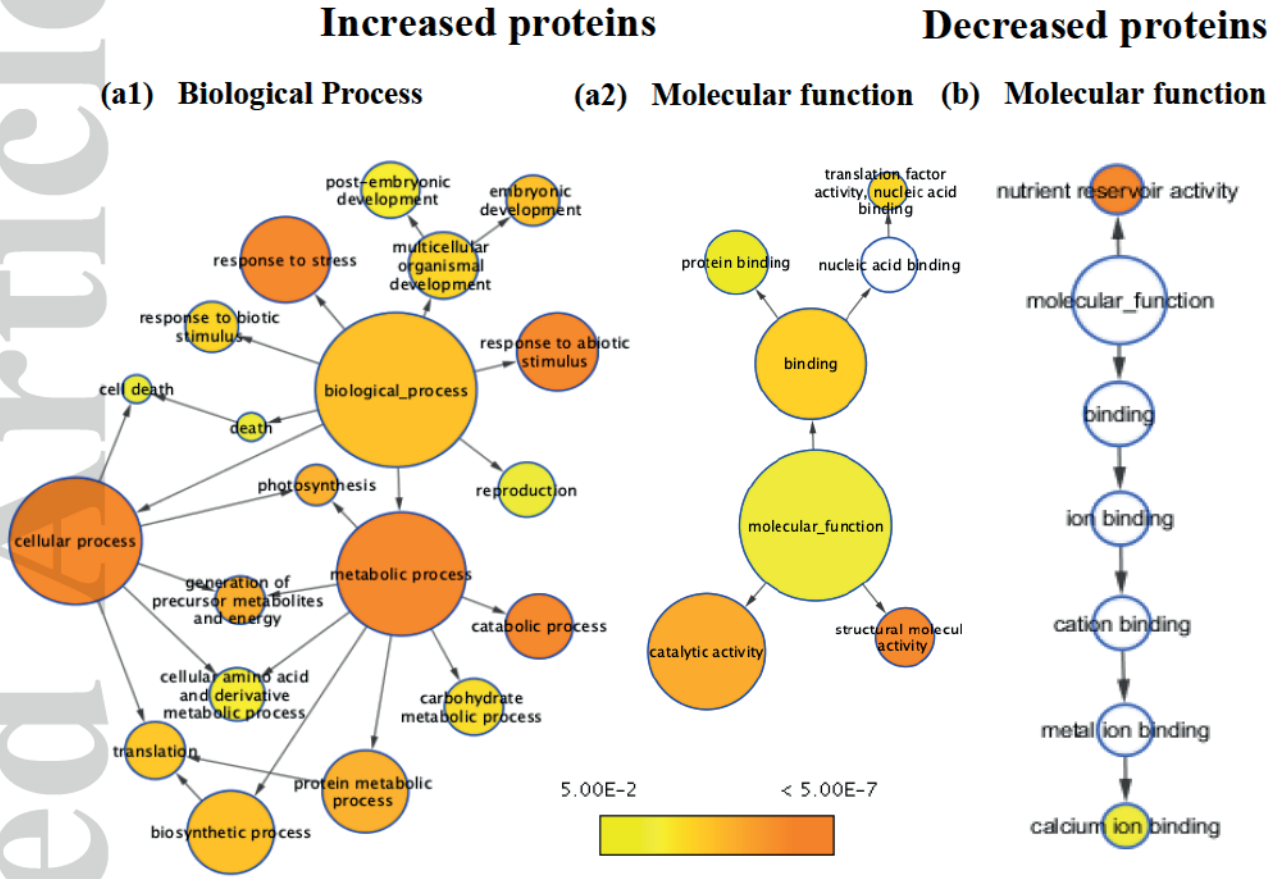

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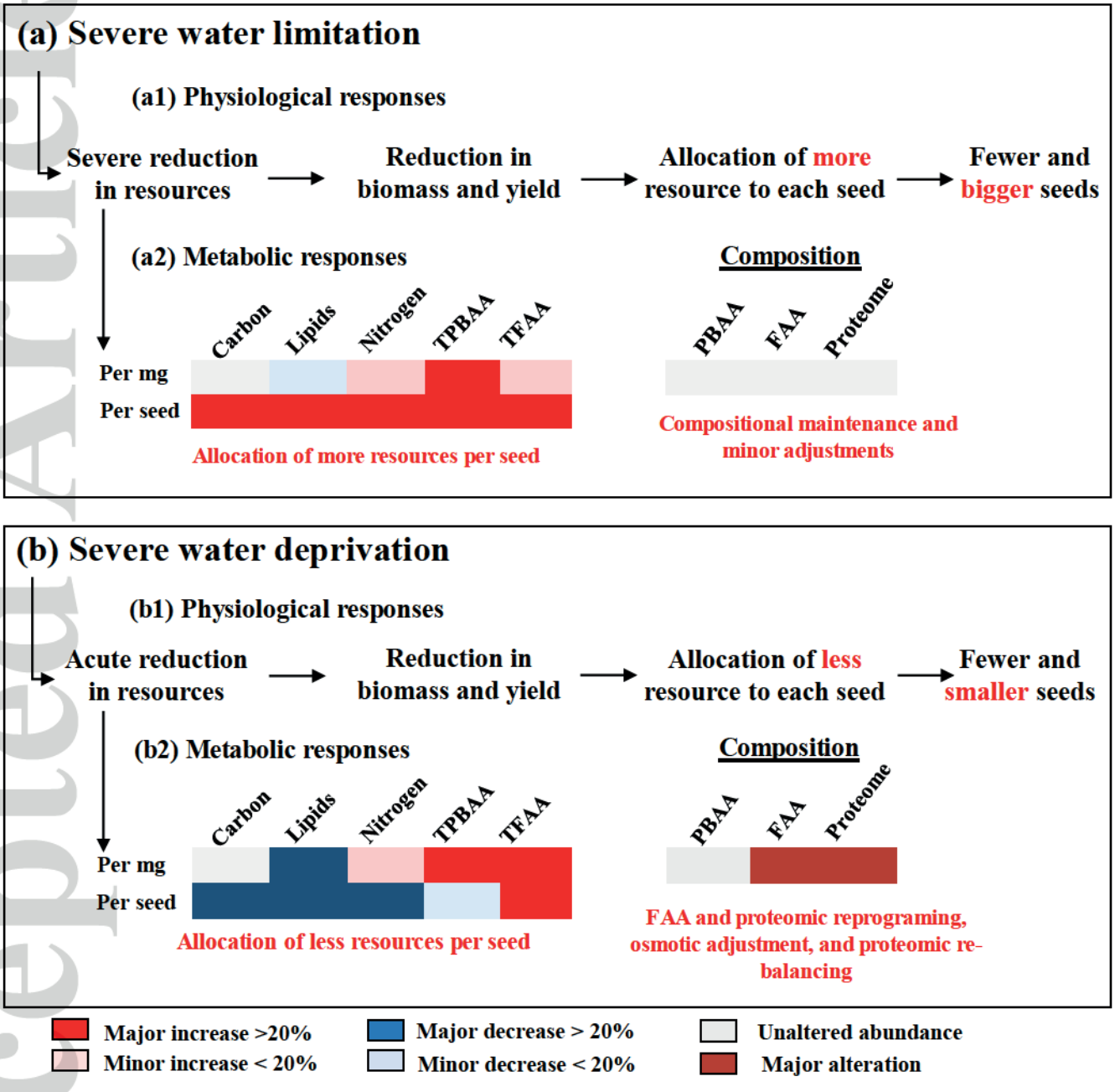

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