Global proteomic analysis of advanced glycation end products in the Arabidopsis proteome provides evidence for age-related glycation hot spots

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Glycation is a post-translational modification resulting from the interaction of protein amino and guanidino groups with carbonyl compounds. Initially, amino groups react with reducing carbohydrates, yielding Amadori and Heyns compounds. Their further degradation results in formation of advanced glycation end products (AGEs), also originating from α-dicarbonyl products of monosaccharide autoxidation and primary metabolism. In mammals, AGEs are continuously formed during the life of the organism, accumulate in tissues, are well-known markers of aging, and impact age-related tissue stiffening and atherosclerotic changes. However, the role of AGEs in age-related molecular alterations in plants is still unknown. To fill this gap, we present here a comprehensive study of the age-related changes in the Arabidopsis thaliana glycated proteome, including the proteins affected and specific glycation sites therein. We also consider the qualitative and quantitative changes in glycation patterns in terms of the general metabolic background, pathways of AGE formation, and the status of plant anti-oxidative/anti-glycative defense. Although the patterns of glycated proteins were only minimally influenced by plant age, the abundance of 96 AGE sites in 71 proteins was significantly affected in an age-dependent manner and clearly indicated the existence of age-related glycation hot spots in the plant proteome. Homology modeling revealed glutamyl and aspartyl residues in close proximity (less than 5 Å) to these sites in three aging-specific and eight differentially glycated proteins, four of which were modified in catalytic domains. Thus, the sites of glycation hot spots might be defined by protein structure that indicates, at least partly, site-specific character of glycation.

Protein glycation is a non-enzymatic post-translational modification formed by interaction of lysyl and arginyl residues with carbonyl compounds (carbohydrates and α-dicarbonyls) (1). In the first step, reducing sugars, aldoses and ketoses, react with free protein amino groups, yielding early glycation products, Amadori and Heyns compounds, respectively (Fig. 1) (2, 3). The early glycation products and their Schiff base intermediates undergo oxidative degradation (glycoxidation) accompanied by formation of advanced glycation end products (AGEs), a heterogeneous group of compounds essentially varying in their structure and stability (4). Alternatively, sugars can autoxidize

3 The abbreviations used are: AGE, advanced glycation end product; aq., aqueous; Argp, argpyrimidine, N5-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-l-ornithine; Asc, l-ascorbate; BAC, boronic acid affinity chromatography; CEA, N5-(carboxyethyl)lysine; CML, N5-(carboxymethyl)lysine; CEL, N5-(carboxyethyl)arginine; CMA, N5-(carboxymethyl)arginine; DHA, dehydroascorbic acid; El, electron ionization; GLAP, glyceraldehyde-derived pyridinium compound; Glarg, glyoxal-derived hydroimidazolone, 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine; GOx, glyoxalase; GR, cytosolic glutathione reductase; tret, retention time index; LOOH, lipid hydroperoxide; MG-H, methylglyoxal-derived hydroimidazolone, N5-(4-methyl-4-oxo-5-hydroimidazolon-2-yl)-l-ornithine; MGO, methylglyoxal; MSTFA, N-methyl-N-(trimethylsilyl)trifluoroacetamide; Orbitrap, orbital trap mass analyzer; ROS, reactive oxygen species; RT-qPCR, real time quantitative-PCR; Rubisco, ribulose-bisphosphate carboxylase/oxygenase; TBA, thiobarbituric acid; TH-Pyr, tetrahydropyrimidine, N5-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-l-ornithine; tret, retention time; UPLC, ultrahigh-performance liquid chromatography; XIC, extracted ion chromatogram; TIC, total ion chromatogram.

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The mass spectrometric raw data and spectral libraries associated with this manuscript are available from ProteomeXchange with the accession number PXD006434.

This article contains Table S1-1 to S1-8, Figs. S1-1 to S1-9, Information 1 and 2, and materials.

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under formation of reactive α-dicarboxyls, e.g. glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (5–7), yielding AGEs via interaction with lysyl and arginyl protein residues (so-called “oxidative glycosylation”) (8). The α-dicarboxyls can also be the products of lipid peroxidation, glycolysis, or the Calvin cycle (9–11).

During previous decades, a variety of AGE structures were characterized in human tissues under hyperglycemic conditions (12) and thermally processed foods (13). Thus, formation of 1-((4-amino-4-carboxybutyl)2-imino-5-oxo-imidazolidine (glyoxal-derived hydroimidazolone, Glarg) was observed in protein incubations with GO (14). This product is unstable under alkaline conditions and easily hydrolyzes to form Nα-(carboxymethyl)arginine (CMA) (15). MGO was shown to modify arginyl residues yielding MGO-derived hydroimidazolones (MG-Hs) (16), and 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolonyl)pentanoic acid (MG-H2) and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolonyl)pentanoic acid (MG-H3) were the minor ones (17). The MG-Hs can sequentially react with two further MGO molecules to form first Nα-(5-methyl-4-oxo-5-hydroimidazolone-2-yl)-l-ornithine; Glarg, glyoxal-derived hydroimidazolone, 1-(4-amino-4-carboxybutyl)2-imino-5-oxo-imidazolidine; Pyr, 3-deoxyglucosone-derived pyrraline, 2′-formyl-5′-hydroxymethyl-pyrrolylnorleucine; Argpyr, argpyrimidine, Nα-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-l-ornithine; TH-Pyr, tetrahydropyrimidine, 4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)l-ornithine. Glyceraldehyde-derived pyridinium compound (GLAP) and 2′-formyl-5′-hydroxymethyl-pyrrolylnorleucine (pyrraline) were characterized in incubation mixtures containing glyceraldehyde and 3-deoxyglucosone, respectively (22).

In the human body, AGEs compromise the functional properties of tissue by intensive cross-linking of long-living proteins (23) and by interaction with receptors to AGEs (or related molecules), expressed on the surface of neurons, macrophages, dendritic, endothelial, and muscle cells, and triggering inflammatory response upon binding of AGEs (24, 25). Currently, accumulation of AGEs over an organism’s life span is recognized as a reliable marker of age-related changes in mammals (26). Surprisingly, recently, Sebeckova et al. (27) reported higher contents of CML in the blood plasma of vegetarians in comparison with omnivorous individuals. This raised the following question. Does the raw plant-derived foods already contain high amounts of AGEs? Bechtold et al. (28) in their pioneering work confirmed the presence of glycation products in exhaustive hydrolysates of plant leaf proteins. Continuing these studies, Shimakawa et al. (29) proposed the endogenic sources of AGEs in plants and some corresponding anti-glycative mechanisms protecting the plants from deleterious effects of accumulated AGEs. Because of the high levels of carbohydrates, transission metals, and molecular oxygen in plant tissues, high levels of monosaccharide autoxidation (6) and therefore essential in vivo α-dicarbonyl and reactive oxygen species (ROS) production can be expected. The resulting AGEs might be an important factor of plant protein damage. However, the age-dependent dynamics of these modifications is still unknown, although a “static snapshot” of the plant glycated proteome was characterized recently (30). Therefore, to address the question of...
whether protein glycation can be a marker of aging in plants, we consider a time dimension of AGE formation in Arabidopsis thaliana. Here we describe the age-related changes in plant glycated proteome and address the underlying factors.

Results

Plant growth and experimental setup

During the harvesting period (7th to 12th week), all plants stayed in the prolonged vegetative phase and did not show essential differences in appearance. Generally, only the plants of growth stages 3.5–3.9 (classification according to Boyes et al. (31)) with well-developed rosettes of ~6–8 cm in diameter and 0.5–1.0 g weight were used, although at the end of the 5-week aging period the plants had larger leaves. No visible signs of stress and no inflorescence formation were observed during the experiment.

Characterization of plant redox status

Biochemical analysis revealed age-related alterations of the plant redox status. Thus, the leaf H$_2$O$_2$ levels increased 2-fold

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Figure 2. Time-course curves of the age-related changes in the leaf contents of stress markers, chlorophylls, and antioxidants. Shown are hydrogen peroxide (H$_2$O$_2$, A), LOOH (B), TBA-reactive substances (expressed as malondialdehyde equivalents, C), chlorophylls (D, solid, dashed and dotted lines represent total chlorophyll, its a and b isoforms, respectively), the Asc/DHA and GSH/GSSG ratios (E, solid and dashed lines, respectively), the contents of total ascorbate, Asc and DHA (F, solid, dashed, and dotted lines, respectively), as well as total glutathione, GSH, and GSSG (G, solid, dashed, and dotted lines, respectively). The A. thaliana plants were grown for 7–12 weeks under 8-h light/16-h dark cycle. All analyses were performed in three biological replicates (three plants per replicate) and one technical replicate.
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Figure 3. Time-course curves of the age-related changes in the relative expression levels of APX1, GR_

transcripts showed a 2-fold decrease during the 1st week of the experiment (Fig. 3A). The status of the glyoxalase system revealed age-related changes, and the abundance of GLX2 transcripts gradually increased during the second half of the observation period (Fig. 3B). The accumulation of the GLX2 transcripts was accompanied by a slight increase of glyoxalase 2 activity at the 11th week of plant growth (Fig. 3C). Finally, based on the kinetics of the redox status markers, three time points for the analysis of the AGE-modified proteome and primary metabolite were selected in the beginning, middle, and the end of the observation period (7, 9, and 12 weeks after germination, respectively).

Metabolite profiling

A GC-MS-based profiling of primary polar metabolites of A. thaliana leaves revealed 51 species (supplemental Table S1-1) identified by retention times and indices (calculated on the basis of analysis of alkane mix (supplemental Fig. S1-1 and supplemental Table S1-2); carbohydrate identification relied on \(^{13}C\) NMR of standard mix (supplemental Fig. S1-2 and supplemental Table S1-3) as described under “Experimental procedures”) and spectra similarity and represented by 31 carbohydrates, 14 amino acids, including 10 proteinogenic ones (Ala, Gly, Val, Ile, Pro, Ser, Thr, Asp, Glu, and Phe) and 5-oxoproline, as well as three non-proteinogenic ones (ornithine, \(\beta\)-alanine, and \(\gamma\)-aminobutyric acid), five di- and tricarboxylic acids (succinic, fumaric, malic, glutaric, and citric acid), and one polyamine (putrescine). Ten compounds (sucrose, mannose, galactose, fructose, gluconic acid, arabino, xylose, erythronic acid, malic acid, and glycine) showed a 2.4–12.5-fold abundance increase over the aging period (i.e. in comparison with 7-week-old plants metabolically characterized earlier (30), t test, significant at the confidence level of \(p \leq 0.05\), among them accumulation of five compounds was also confirmed with Benjamini-Hochberg corrected \(p \leq 0.05\), Fig. 4A). In contrast, no age-related changes in GO and MGO contents were observed (Fig. 4B). The levels of GO were 2.5–3.0-fold higher in comparison with those of MGO.

Protein isolation and tryptic digestion

The protein extraction procedure was efficient and reproducible; the overall protein recovery after the gel filtration and ultrafiltration steps was 69.8 ± 5.0%. The SDS-PAGE revealed a good precision of the protein determination in the resulting extracts (relative standard deviation = 17.9%). The efficiency of the tryptic digestion was higher than 95%, as the Rubisco large subunit band was not visible in the SDS-polyacrylamide gel electropherograms of corresponding digest. Thus, assuming that Rubisco content was at least 20% of the total leaf protein, the sensitivity of the Coomassie staining was better than 30 ng of loaded protein per band (30).

Identification of AGE- and Amadori/Heyns-modified proteins

Altogether, 511 AGE-modified proteins (529 tryptic peptides and 705 AGE sites) and 194 early glycated proteins (196 peptides containing 289 Amadori/Heyns-modified lysyl residues) were identified. The AGE patterns were dominated by argin-
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In the spectra of the supplemental Table S1-4 entries, respectively, see Table 1). The functional annotation of these species revealed 12 groups (5 and 10 for 9- and 12-week-old plants, respectively) dominated by the molecules involved in transcriptional regulation, transport, and protein metabolism (4, 3, and 3 entries, respectively, see Table 1).

Age-related changes in the early glycated proteome

In total, 147 of 194 early glycated proteins (149 peptides/181 modification sites) were detected at all time points (198 sites were earlier reported for 7-week-old plants (30)). Only 10 age-specifically glycated Amadori/Heyns-modified proteins (mostly representing triose-, tetrose-, and pentose-derived products, Table 1) were discovered in the 9th and 12th week harvests, and 17 species only 12 weeks after plant germination (16.5% of the early glycated proteome). The corresponding MS/MS data used for peptide identification are summarized in supplemental information 2. The functional annotation of these species revealed 12 groups (5 and 10 for 9- and 12-week-old plants, respectively) dominated by the molecules involved in transcriptional regulation, transport, and protein metabolism (4, 3, and 3 entries, respectively, see Table 1).

Age-related changes in the AGE-modified proteome

The absolute majority of the annotated AGE-modified proteins (494 of 511) was detected in all age groups (692 sites were earlier reported for 7-week-old plants (30)). Remarkably, only one of these sites (carboxymethylated Lys595 in the voltage-gated chloride channel protein, AT5g26240) was previously detected to be Amadori/Heyns-modified (triose-derived modification in the peptide EACCAMQSQ(KTriosyl)VISLPR601). Only six proteins containing a total of nine AGE-modification sites were age-specifically glycated (Table 2, the MS/MS information is provided in supplemental information 2).

Relative quantification of individual AGE modification sites

The quantitative analysis revealed distinct age-related changes in the abundances of the individual AGE-containing tryptic peptides (Fig. 6). Thus, 96 AGE residues in 71 glycated proteins (represented by 72 tryptic peptides) showed at least a 1.5-fold change in their abundance (for identification details see supplemental Tables S1-4 and S1-5). Among this number, 45 and 61 glycation sites (33 and 43 proteins, respectively) were significantly up-regulated after 9 and 12 weeks, respectively (t test, significant at the confidence level of \( p \leq 0.05 \)), at 12th week the enhanced glycation rates of 19 proteins were additionally confirmed by Benjamini-Hochberg corrected \( p \leq 0.05 \) (supplemental Table S1-4). Furthermore, two residues in one protein and 11 sites in 10 proteins were significantly down-regulated at these time points, respectively (Fig. 6A and supplemental Table S1-4). The differentially glycated sites are dominated by CML and MG-H, whereas CEA did not show quantitative changes at 9 weeks after germination (Fig. 6A). Although most of the age-responsive AGE-modified proteins demonstrated 1.5–10-fold changes, some CML-, CEA-, CMA-, and Argpyr-modified sites were up-regulated up to 28-fold (Fig. 6B, for identification details see supplemental Tables S1-4 and S1-5).
The observed modifications mostly originated from GO, sugar, or the products of Amadori/Heyns degradation, whereas MGO was the less prominent glycation agent. Among the down-regulated sites, the abundances of CMA514 and CEA516, representing leucine-rich repeat receptor-like serine/threonine-protein kinase, decreased most significantly (up to 3-fold).

The analysis of the sequence consensus motifs performed for the differentially AGE-modified arginyl residues revealed a high probability of Arg at positions 4, 6, and 10 and Lys at positions 2 and 8.
### Table 1

The unique age-related Amadori/Heyns-modified proteins identified in the tryptic digests obtained from the 9- and 12-week-old A. thaliana plants

| Nr | Protein name | Accession number | Genomic identifier | Peptides identified | Peptide sequence | Modified sites | $m/z$ | XCorr | $t_r$ | Plant age, weeks |
|----|--------------|------------------|-------------------|--------------------|-----------------|----------------|-------|-------|--------|-----------------|
| 1  | Transketolase-2, chloroplastic | F4W47 | AT2G45290 | 7 | GK$_{Tryosyl}$SIGDITFGASAPAGK | K$_{208}$ | 839.94 | 2 | 2.02 | 35.4 | 9 |
| 2  | Bifunctional monothiol glutaredoxin-S16, chloroplastic | Q817F6 | AT2G38270 | 3 | VPPGNK$_{Tryosyl}$SGNNTFVK | K$_{208}$ | 780.9 | 2 | 2.16 | 41.4 | 9 |
| 3  | Cell redox homeostasis | T4B21.20 protein | Q9ZS86 | - | RETTESAM$_{Tryosyl}$ETVSYSKQAK$_{Tryosyl}$ LLR | M$_{14}$ K$_{14}$ | 828.1 | 3 | 2.58 | 28.3 | 32.1 |
| 4  | Amino acid metabolism | Ca$^{2+}$-dependent plant phosphoribosyltransferase family protein | Q3M666 | AT3G61720 | 1 | MK$_{Phospho}$LTDQAYHVAEETQYS SDNR | C$_{255}$ | 931.4 | 2 | 2.75 | 32.1 | 9 |
| 5  | Protein metabolism | Elongation factor 1-alpha 2 | Q94AD0 | AT1G07930 | 8 | ERGIDIADL[Keu$]_{Tryosyl}$IETTK | M$_{28}$ K$_{28}$ | 688.39 | 3 | 2.53 | 31.2 | 32.1 |
| 6  | Subtilisin-like protease SBT4.11 | Q9F1G1 | AT5G59130 | 1 | [Keu-OH]$^{2+}$KICGACGGKNCFTC$_{Phospho}$ | C$_{20}$ K$_{20}$ | 962.93 | 2 | 1.96 | 32.2 | 32.1 |
| 7  | Putative F-box only protein 10 | Q89YD0 | AT1G51290 | 1 | LC$_{44}$NTTSKIKIWLTK$_{Phospho}$ | C$_{20}$ | 928.52 | 2 | 2.09 | 43.0 | 32.1 |
| 8  | Transcriptional regulation and DNA/RNA processing | 3'-5'-Exoribonuclease family protein | Q9ZU14 | AT1G60080 | 1 | RKLTLK$_{Tryosyl}$NIPFSLTCLHLK$_{Tryosyl}$ | K$_{220}$ K$_{220}$ | 777.11 | 3 | 2.81 | 35.6 | 32.1 |
| 9  | Copia-like retroelement polyprotein | O82196 | AT2G19840 | 1 | MANNSFSEVK$_{Tryosyl}$GIGK$_{Tryosyl}$ | K$_{200}$ | 858.41 | 2 | 2.08 | 25.5 | 32.1 |
| 10 | MAR-binding filament-like protein 1 | Q7V1Z5 | AT3G16000 | 1 | K$_{Phospho}$TVLSLNKEVK$_{Tryosyl}$ | K$_{234}$ | 731.92 | 2 | 1.91 | 31.3 | 32.1 |
| 11 | ATP-dependent DNA helicase homolog REC6, chloroplastic | Q9VZG0 | AT2G01440 | 1 | K$_{Phospho}$FPKY$_{Glu}$NCGLHLGRMK | K$_{235}$ | 970.5 | 2 | 2.17 | 34.0 | 32.1 |
| 12 | Cysteine-rich repeat secretory receptor-like protein kinase 34 | Q9LRLK3 | AT3G22040 | 1 | EKLHAMVQCO$_{Glu}$NKH$_{Phospho}$DLADCK$_{Tryosyl}$ | C$_{190}$ | 1076.99 | 2 | 2.06 | 38.5 | 32.1 |
| 13 | Transport | Sugar transport protein 14 | Q9SX48 | AT1G77210 | 1 | KQMLHNLTD[Oxalactone]C$_{Glu}$K$_{Tryosyl}$ | K$_{278}$ Y$_{278}$ | 844.35 | 2 | 2.32 | 1.4 | 32.1 |
| 14 | ABC transporter G family member 41 | Q7PC83 | AT4G15215 | 1 | LK$_{Phospho}$FI$^{2+}$VPDPIDAYMK | K$_{200}$ | 969.99 | 2 | 1.99 | 34.2 | 32.1 |
| 15 | Copper transport family protein | Q9LTE8 | AT5G52600 | 1 | RDMV$_{Tryosyl}$FEVIEFSGTSVELK$_{Phospho}$ | M$_{143}$ K$_{143}$ | 828.1 | 3 | 2.60 | 28.3 | 32.1 |
| 16 | Cell organization | Myosin-15 | Q9SMY9 | AT4G33200 | 1 | SILTREGIK$_{Phospho}$ | K$_{300}$ | 468.95 | 3 | 2.68 | 31.8 | 32.1 |
| 17 | Glutathione S-transferase U6 | Q98ZM9 | AT2G29440 | 1 | KFPEYNK$_{Phospho}$[Kyn-OH]VKK | K$_{30}$ W$_{30}$ | 839.94 | 2 | 2.01 | 35.4 | 32.1 |
| 18 | Detoxication | Glutathione S-transferase U6 | Q92W26 | AT2G29440 | 1 | KFPEYNK$_{Phospho}$[Kyn-OH]VKK | K$_{30}$ W$_{30}$ | 839.94 | 2 | 2.01 | 35.4 | 32.1 |
| 19 | Disease resistance | Thionin-2.2 | Q42597 | AT5G36910 | 1 | ACSSVC$_{Glu}$TGOSTAAVK$_{Phospho}$ | C$_{233}$ K$_{32}$ | 862.37 | 2 | 1.93 | 30.1 | 32.1 |
| 20 | Disease resistance protein (TIR-NBS-LRR class) family | Q9F2H29 | AT5G40100 | 4 | SLIDMDSNNDEVRMIG[Kyn]GM$_{63}$GGGKTTIAK$_{Phospho}$ | W$_{314}$ M$_{136}$ K$_{226}$ | 1135.56 | 2 | 2.56 | 28.3 | 32.1 |
positions 2 and 7 N-terminally from the glycated residues, whereas no hydrophobic residues were characteristic for this part of the consensus (Fig. 7A and supplemental Fig. S1-5A). In contrast, C-terminally from the glycation site, the two proximal positions were often occupied by Leu, whereas Arg, Lys, and Thr appeared at a higher distance from the glycation site. The consensus sequence for the lysine-derived AGEs was similar, and it strongly dominated with charged amino acids at the N-terminal side of the glycation site (Fig. 7B and supplemental Fig. S1-5B). The probability of Cys, Met, Tyr, and Trp in close proximity to both types of glycation sites was the lowest (Fig. 7 and supplemental Fig. S1-5).

To clarify whether the increased abundances of specific glycation sites were caused by enhanced AGE formation or age-related changes of the expression levels, the differentially glycated proteins were quantified on the relative basis using at least one proteotypic peptide per protein. To select such peptides, the raw data were re-searched against a FASTA file containing only the sequences of the identified differentially glycated proteins. Among the 14 proteins annotated, the un-
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Figure 6. Age-related quantitative changes in *A. thaliana* glycated proteome. Numbers of individual AGE-modified sites (sorted by individual AGE classes) demonstrate significant abundance changes of at least 1.5-fold (t test, \( p < 0.05 \)) identified in proteins from 9- (white) and 12- (gray)-week-old *A. thaliana* plants (A), and the degree of these quantitative alterations expressed in scale of fold changes in 9- (B), and 12-week-old plants (C). In detail, the AGE-carrying sites (TH-Pyr, dark brown; Argpyr, light brown; CEA, pink; MG-H, red; Glarg, light blue; CMA, green; CML, yellow; CEL, white; pyrraline, dark blue; GLAP, purple) are distributed in \( n \)-fold change groups according their accumulation in 9- (B) and 12 (C)-week-old plants. The full names of the AGE modification are indicated in Fig. 1. The upper panels in A–C represent the count of modified sites, which increased in their abundance, and the lower panel shows glycated sites with decreased abundance.

Figure 7. Consensus sequences of *A. thaliana* proteins (25 residues up- and downstream) built for 51 arginyl (A) and 24 lysyl (B) AGE-modified residues (\( R^* \) and \( K^* \), respectively) demonstrating significant changes in glycation rates. The consensus analysis was performed with the WebLogo Beta software tool. The position-specific occurrence frequencies of individual residues are expressed as informational unit bits. The bit axis was zoomed to provide better access to the residues in close proximity to the glycation site. The unprocessed graphs are available as supplemental Fig. S1-5.
tyties of 12 species were unchanged (Table 3), whereas the abundances of the corresponding AGE-peptides were 1.5–22-fold increased (Table 3), unambiguously indicating enhancement of advanced glycation (but not expression up-regulation) as the reason for the observed abundant increase of specific glycation sites.

After 12 weeks of growth, the most essential age-dependent increase of glycation rates was observed for DNA-binding protein (encoded by \textit{AT3g45830}) and phospholipid: diacylglycerol acyltransferase 1 (encoded by \textit{AT5g13640}) (Table 3). The other age-dependently AGE-modified proteins were involved in photosynthesis, protein metabolism, vesicular trafficking, transcriptional regulation, signaling, cell organization, cell wall remodeling, or were of unsigned function (1, 1, 1, 4, 1, 2, 1, and 1 entries, respectively). The up-regulated AGE sites were mostly represented by CML, CMA, and MG-H (Fig. 8A). Most of the hydroimidazolones accumulated during the first half of the observation period (Fig. 8B), indicating MGO as the main glycation agent in that period (Fig. 8D). Later, however, the contents of CML- and CMA- modified peptides dramatically increased (Fig. 8C), indicating the impact of GO and early glycation in the AGE formation (Fig. 8E). Remarkably, the changes in AGE patterns were not accompanied by the alterations in the carboxylated proteome; the Western blotting analysis did not reveal any age-related changes in the patterns of dinitrophenyl-hydrazine-reactive proteins.

**Structural aspects of AGE formation**

The analysis of the sequence motifs in the sites sensitive to the age-related glycation (6 age-specific and 14 age-dependently up-regulated glycation sites, comprising 13 arginine- and 13 lysine-derived glycation modifications) revealed Lys, Arg, Glu, Asp, and Leu as the most frequently occurring residues in close proximity to the AGE-modified residues, whereas Trp, His, Cys, and Met demonstrated negligible occurrence in glycation motifs (Fig. 9 and supplemental Fig. S1-6). Moreover, the glycation consensus motif had a clearly periodical character. Thus, for the arginine-derived AGES, the favorable residues occurred in the positions 3, 7, 11, 14, 20, and 4, 8, 13, 16, 21 up- and downstream the glycation sites, respectively (Fig. 9A and supplemental Fig. S1-6A), whereas for the lysine modifications these positions were 1, 6, 11, 19, 21 and 6, 7, 9, 10, 12, 13, 16, 17, 22, 23 (Fig. 9B, supplemental Fig. S1-6B). These patterns might indicate preferential glycation in α-helical domains.

Analysis of the protein sequences in the domain databases revealed glycation in the catalytic domains of the age-specifically glycated proteins β-carboxy anhydrase 2 and ACT domain-containing protein ACR9, whereas fucosyltransferase 7 was modified at Lys^693 (CML) and Lys^694 (CML) in the close proximity to C terminus of the main functional domain (supplemental Table S1-6). In four proteins, shown to be age-dependently glycated (namely phospholipid: diacylglycerol acyltransferase 1, E3 ubiquitin-protein ligase ORTHRUS 1, protein SPG1-RELATED 4, and rhamnogalacturonate lyase family protein), the AGE modification sites were localized within their functional (catalytic) domains. In four other proteins (ubiquitin C-terminal hydrolase 12, dynamin-related protein 1E, P-loop containing NTP-hydrolases superfamily protein, and uncharacterized AT4G38062 protein), the glycated sites were located in conservative domains, which, however, were not responsible for catalytic function of the proteins but might be involved in interactions with other cellular components.

Homology modeling performed with the age-dependently glycated proteins resulted in 17 successful models (supplemental Table S1-6). Because all sequences have a similarity to the best-suited template for homology modeling, higher than 30% all the models were of sufficient quality. Evaluation of the models revealed 14 proteins with AGE modifications in the molecule parts essential for enzymatic catalysis, activity regulation, and, in the majority of cases, interaction with other cellular macromolecules, i.e. in the surface regions (Fig. 10). Twelve of these proteins revealed the models containing the glycation sites of interest. The manual search for structural consensus motifs revealed aspartyl and glutamyl residues in the close proximity to lysyl and arginyl side chains in 10 cases, typically in a distance of less than 5.0 Å (Fig. 10).

**Discussion**

**Experimental setup**

In plant tissues, long-living proteins are exposed to various carbohydrates, present at high contents on the background of essential equilibrium concentrations of ROS. Therefore, glycation of such proteins with the products of sugar autoxidation seems to be rather probable (30). To address the question of whether this phenomenon impacts plant aging (as is known for mammals (26, 35)), a suitable model is required. Indeed, to distinguish molecular aging from regular senescence-related ontogenetic changes, experimental plants need to be observed within one developmental stage. It is well-known that under the long day conditions (16-h light/8-h darkness cycles), \textit{A. thaliana} plants complete their life cycle in 7–8 weeks (31). However, the switch to the short day (8-h light/16-h darkness cycles) results in essential prolongation of vegetative phase (36). Generally, the beginning of the vegetative phase is characterized by active growth processes, accompanied by high carbon and nitrogen uptake and protein synthesis (37). Later, when the metabolic capacity of photosynthetic machinery is achieved, the processes of protein synthesis and degradation reach equilibrium. Thereby, protein turnover remains at a consistently low level (38). Thus, a short day experimental setup suits well the study of age-related changes in long-living \textit{Arabidopsis} proteins.

**Biochemical characterization of aging**

Generally, ROS-mediated protein damage due to the acceleration of monosaccharide autoxidation and advanced glycation always accompanies aging (8). In this context, the contents of hydroperoxides and hydroxyl radical (OH·), i.e. the principal ROS involved in sugar autoxidation (5), might give insight in the rates of this process. However, although H₂O₂ and lipid hydroperoxides (LOOHs) are relatively stable species, OH· is a quite reactive ROS and only can be detected by the products of its reaction with biomolecules (39, 40). In our experiments, the contents of H₂O₂, LOOHs, and dicarboxyls (malondialdehyde) increase in parallel to the up-regulation of several sugars known...
### Table 3

Results of the label-free quantification of differentially age-dependently glycated proteins and corresponding glycation sites

| Nr | Protein annotation | Accession numbera | Genome identifierb | Peptides identified | Peptide sequence | Modified sites | Identification | Quantification/ (fold changes) |
|----|--------------------|-------------------|-------------------|--------------------|----------------|---------------|---------------|-----------------------------|
|    | **Photosynthesis. Light dependent reactions** | | | | | | | |
| 1  | PsPFDI1795-like photosystem II reaction center PsP family protein 5, chloroplast | P82715 | AT5G11450 | 2 | SSNISS[CML]YHAGAS[CML] | K26 R32 | 741.35 | 2 | 2.21 | 30.59 | 3.37 | 4.76 |
| 2  | Phospholipid:di acylglycerol acetyltransferase | Q9FNA9 | AT5G13640 | 2 | AW[c][CMA][GTK][CMA][F] NPSGK | R398 R394 | 891.45 | 2 | 2.65 | 30.56 | - | 14.0 |

|    | **Lipid metabolism** | | | | | | | |
| 2  | | | | | | | | |
| 1  | Ubiquitin carboxyl-terminal hydrolase 12 | Q9FPT1 | AT5G06600 | 2 | Y29AG[MG-H]LM63VKS SKPMDTGK | M113 | 1086.04 | 2 | 2.23 | 27.96 | 6.18 | - |
| 4  | Dynamin-related protein 1E | Q9FNX5 | AT3G60190 | 3 | NTQAPSATLDOYDDGHF | R | 748.86 | 2 | 2.2 | 25.65 | 2.93 | - |
| 5  | E3 ubiquitin-protein ligase ORTHUS | Q9FKA7 | AT3G9550 | 3 | DLSONK[CMA]JNKK | R238 | 665.87 | 2 | 2.29 | 27.82 | 2.36 | 3.72 |
| 6  | PHD finger-containing protein | F4RJ4 | AT1G77800 | 4 | EAMNIEFK | R126 | 867.9 | 2 | 2.4 | 24.96 | 1.48 | - |
| 7  | DNA-binding protein-like | Q9LU7 | AT3G45830 | 2 | EPGQ[Oxaloacetate][IS][CMA][P] GK[CML] | M161 | 688.84 | 2 | 2.38 | 23.52 | - | 22.1 |
| 8  | HD zinc finger-containing protein | F4KE5 | AT3G16680 | 2 | NL[Oxaloacetate][CML][P]LVNDN MIDCML[K]ANNK | R292 | 1006.04 | 2 | 2.23 | 28.18 | 3.68 | - |
| 9  | Cytoplasmic rRNA 2-thiolation protein 1 | O64B62 | AT2G44270 | 2 | NCMJUKAVASRLCNC[c]CNC[c]NLR | K6 | 969.09 | 2 | 2.29 | 33.8 | - | 4.48 |

|    | **Transcriptional regulation** | | | | | | | |
| 10 | Protein SPA-RELATED 4 | Q9HB7 | AT1G53090 | 2 | TKANICC[c][CMA][QFQ][SET] GIC[MG-H]SLAFSGADHK | C232 | 1068.98 | 2 | 2.25 | 24.36 | - | 2.25 |

|    | **Cell organization** | | | | | | | |
| 11 | P-loop containing nucleoside triphosphate hydrolases superfamily protein | F41T9 | AT1G55550 | 2 | K[M][Arg][y]NLTNEK | R141 | 728.39 | 2 | 2.24 | 29.85 | - | 10.4 |
| 12 | WEB family protein | F4ISY0 | AT2G38370 | 2 | DLSQA[GLAP][M]LNC[c]CAM[c]KIAISR | K140 | 987.01 | 2 | 2.38 | 28.57 | - | 1.9 |

|    | **Cell wall** | | | | | | | |
| 13 | Rhamnogalacturonase-base family protein | Q93Z09 | AT4G24430 | 2 | LENVQKSCCTY[K][MG-H] | Y396 | 826.42 | 2 | 2.31 | 22.13 | 1.74 | 4.18 |

|    | **Not assigned** | | | | | | | |
| 14 | Uncharacterized protein | PO823 | AT4G38062 | 2 | LJ[CML][AELE][Q][QQNL][M][M][M][E][E]SS[L][S][Q] | K737 | 757.41 | 2 | 2.32 | 27.39 | 2.15 | 3.31 |

*a* UniprotKB protein accession numbers are given.  
*b* Arabidopsis genome initiative identifiers in TAIR database are given.  
*Protein label-free relative quantification for samples from 9- and 12-week old plants in comparison to 7-week-old ones.  
*n-fold changes observed in 9-week-old plants in comparison with 7-week-old ones.  
*m-fold changes observed in 12-week-old plants in comparison with 7-week-old ones.  
*All differences in peptide abundance were statistically significant (p ≤ 0.05). C[CAM]cysteine carbamidomethylation; C[c]CNC[c][CMA][QFQ][SET]cysteine sulfenic and sulfinic acid, respectively; M[c]L[c]methionine sulfoxide and sulfone, respectively; Y[c]DOPA, 2,4-dihydroxyphenylalanine; W[c]hydroxy-N-formylkynurenine; m/z mass to charge ratio; tε retention time; ε charge. Modified residues in peptide sequences are in brackets. The AGE modifications in bold font are located in a predicted functional domain of the protein. All analyses were performed in three biological replicates (three plants per replicate) and one technical replicate.
to be potent glycation agents (Figs. 2, A–C, and 4A) (1, 30). Hence, enhancement of dicarbonyl production and increased advanced glycation rates can be expected. However, a relatively low increase of lipid hydroperoxide contents (Fig. 2, B and C) indicates scavenging of this ROS by the cellular antioxidant systems (Fig. 2, E–G).

As Asc and GSH are involved in such protective systems (41), the increase of total ascorbate levels (Fig. 2F) can be explained by its higher consumption for the reduction of overproduced hydroperoxides. The moderate age-dependent decrease of GSH/GSSG ratio and total glutathione contents (Fig. 2, E and G) might indicate depletion of GSH due to scavenging of OH·, decrease of GCrept leaf transcript levels (Fig. 3A), and involvement of GSH in detoxication of α-dicarboxyls by the glyoxalase system (42). The latter assumption is confirmed by the higher expression levels and activity of GLX2 (Fig. 3, B and C) without any effect on GO and MGO leaf contents (Fig. 4B). However, as the Asc/DHA ratio and the levels of APX1 (APX1 is cytosolic ascorbate peroxidase) expression remained unchanged (Figs. 2E and 3A), it can be concluded that the antioxidant capacity of the plants was not overwhelmed during the analyzed period.

**Age-related changes in the glycated proteome**

Recently, using the methods of LC-based bottom-up proteomics, we identified 502 glycated proteins in 7-week-old *A. thaliana* leaves (30). That study, however, provided only a “snapshot” of the plant glycated proteome. To extend our knowledge and understanding of glycation in plants also in the temporal dimension, we considered here this earlier published dataset (30) complemented with two later time points relying on the established set of AGEs (14–22). Surprisingly, only 1.2% of the annotated proteins were identified solely at later time points (9th and 12th week, Table 2). Thus, the distribution of the detected AGEs by classes was minimally affected by aging and resembled previous observations (30). Hence, although the glycated proteome is represented by hundreds of proteins, highly complex in terms of half-lives and compartmentalization, it is conservative in time, at least under the highly controlled environmental conditions used for these experiments.

Surprisingly, quantitative effects of aging on the plant glycated proteome were much more pronounced (supplemental Table S1-4). Indeed, 14% of the total glycated proteome was age-dependently up-regulated (p ≤ 0.05, supplemental Tables...
S1-4 and S1-5), which could be explained, at least partly, by the site-specific increase of glycation levels, which was confirmed by label-free quantification of corresponding proteins (Table 3). Thus, the part of the proteome that is susceptible to glycation is rather conservative, and only a limited pattern of proteins is affected by aging. This observation clearly indicates the existence of “glycation hot spots” in the plant proteome. This phenomenon is already known for individual mammalian proteins (e.g. serum albumin) (43) and recombinant antibodies (44). The existence of such hot spots can be explained not only by longer half-lives and different compartmentalization but also by different amino acids surrounding the polypeptide chain and the three-dimensional structure of protein molecules (45).

As only few new amino acid residues become Amadori- and Heyns-modified with plant age, it can be concluded that the mechanisms of the AGE formation are not affected by this factor and generally resemble those described in our previous work, i.e. the impact of glycoxidation on the patterns of age-related AGEs is negligible (as the AGE-modified sites are not detectable as Amadori/Heyns products), whereas monosaccharide autoxidation is the main source of AGEs (30). However, the increasing contents of reducing sugars might affect the glycation rates and glycated proportion of specific AGEs at particu-
lar residues. Indeed, despite its non-enzymatic mechanisms, advanced glycation is also a catalyzed process; the residues, localized close to arginine and lysine, can affect their reactivity toward reactive carbonyls and the stability of already formed adducts. Such neighboring group effects with acid/base or redox catalytic function are well-known (46, 47). Thus, glycation by lysines is modulated by basic and acidic residues located in close sequence to structural proximity from a glycation site (48). Indeed, in our previous work on peptide glycation models, we demonstrated that acidic residues increase the stability of aldol-derived hydromidazolones (as well as their hydrolysis products) and CML, whereas basic residues enhance degradation of AGES or their Amadori precursors (4, 18). In this context, the existence of glycation-specific sequence or structural consensus motifs can be assumed.

The most comprehensive study of glycation consensus sequence motifs was reported by Zhang et al. (50) for plasma and erythrocyte proteomes. The characteristic motifs, identified by the authors with the WebLogo software tool, were dominated with uncharged residues, whereas the occurrence of His, Tyr, Met, Trp, and Cys near the glycation sites was the lowest. The results are in agreement with our data calculated for the Amadori/Heyns sites specific for plant aging (Table 1); the corresponding motifs, besides uncharged residues, contained also acidic and basic ones and fewer numbers of His, Tyr, Met, Trp, and Cys (supplemental Fig. S1-7). Interestingly, statistically analysis of glycated lysines of human proteins showed more acidic residues to be located C-terminally from the glycation site, whereas the basic ones were more characteristic for the N-terminal side. Based on this observation, an artificial neuronal network-based predictor of glycation NetGlycate-1.0 (http://www.cbs.dtu.dk/services/NetGlycate/output.php) was proposed (51). However, this tendency was not observed in our study.

To compare the sequence motifs characteristic for plant AGES (Figs. 7A and 9A and supplemental Figs. S1-5 and S1-6) with those in mammalian proteins, we processed the published datasets acquired for plasma AGE proteome (33, 52) with the WebLogo software tool (supplemental Figs. S1-8 and S1-9). The comparison for arginine- and lysine-derived AGE sites revealed similar patterns of frequently occurring charged (Lys, Arg, Glu, and Asp) and uncharged residues (Leu, Val, Ala, and Pro) in close proximity to the glycated sites in plant and mammalian proteins. The residues, which were less frequent in the Amadori/Heyns-modified motifs, were less represented in the AGE-modified ones as well. In both plant and human plasma sequence motifs, the most conservative residues were localized N-terminally relative to the glycated sites. It was the directly adjacent or the 2nd to 6th residue from an AGE adduct in plant and plasma protein sequence motifs, respectively (Figs. 7A and 9A and supplemental Fig. S1-5, S1-6, S1-8, and S1-9). This remotely indicates some similarity of glycation preferences and mechanisms between plants and mammals. This can be explained by the fact that, in both cases, glucose is the major sugar (although in plants it is only a small part of a more varied carbohydrate pattern) and can be involved in antioxidative processes.

Remarkably, besides sequence moieties, characteristic structural motifs can be proposed in the age-specifically and age-dependently glycated proteins. The presence of glutamyl and aspartyl residues in close spatial proximity (typically less than 5.0 Å) from the corresponding AGE modification sites (Fig. 10 supplemental Table S1-6) was in agreement with the kinetics of AGE formation and degradation obtained from model glycation experiments with synthetic peptides (4, 18), as well as from observations done with the patterns of early glycation (45). Generally, the presence of negatively charged amino acid residues in close proximity to the glycation sites can be explained by the known AGE formation mechanisms. Thus, anionic residues may stabilize the basic hydromidazolones (MG-H and Glarg) and increase the times required for their degradation and further conversion into other AGES (e.g. MG-H to Argpyr). The impact of these residues in the CML formation in plants is difficult to explain. Indeed, in the glycoxidative pathway these anionic residues might enhance deprotonation, required for the keto-enol isomerization of a Amadori/Heyns-derived α-dicarbonyl intermediates before its β-dicarbonyl cleavage. However, because of the low impact of glycoxidation in glycation of plant proteins (30), the relevance of this mechanism for plants is questionable, and alternative mechanisms are currently under consideration in our group.

Functional aspects of the age-related glycation

The functional data obtained are in agreement with the previous results (30, 53) and confirm the proteins involved in the transcriptional regulation as the primary targets of advanced glycation (Table 3). The molecules participating in protein metabolism represented another large group. Thus, E3 ubiquitin-protein ligase ORTHRUS 1 was carboxymethylated in an age-dependent manner at arginine 330 (Table 3). As this residue is situated in the SRA-YDG domain (responsible for the association with histone methyltransferases), this modification might not only directly affect the protein function (E3 ubiquitin-protein ligase activity (54)), but also influence methyltransferase activity. Similarly, SPA1-RELATED 4, the negative regulator of the phytochrome A-mediated photomorphogenesis (55), was found to be glycated by Arg632 (MG-H) in the 4th WD repeat and may thereby affect protein-protein interactions. As the SPA proteins are the key players in light signaling, they interact with transcription factors LONG HYPOCOTYL 5 (HY5), E3 ubiquitin ligase COPI (CONSTITUTIVE PHOTOMORPHOGENIC 1), floral inducer CONSTANS (CO), blue-light receptor cryptochrome 1 (CRY1), and others and regulate elongation growth and flowering time (56). Thus, interaction of MGO with Arg632 in SPA4 can interfere with photomorphogenesis regulation and affect plant growth. According to the data of homology modeling and domain database search, further functionally affected molecules might be the ACT domain-containing protein ACR9, phospholipid:diacylglycerol acyltransferase 1, and rhamnogalacturonate lyase family protein (Tables 2 and 3 and supplemental Table S1-6).

Although only a few unique stress-specific glycation sites were detected, they represent important enzymes. Thus, glyca-
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The seeds of wild-type A. thaliana (Columbia 1092) were obtained from the plants grown in-house. The seeds were planted in wet soil/sand mixture, and the plants were grown in fully climatized growth chambers at 22 °C (day) and 18 °C (night), 60% relative humidity, under short day conditions with an 8-h light (150 ± 2.5 μmol photons m−2 s−1)/16-h dark cycle for 12 weeks. The plants were harvested at 7, 7.5, 8, 8.5, 9, 9.5, and 10–12 weeks (n = 9 per time point pooled in groups of three). The leaf rosettes were ground in liquid nitrogen by a Mixer Mill MM 400 ball mill in stainless steel grinding jar (25 ml) with 25-mm diameter stainless steel balls (Retsch, Haan, Germany) at a vibration frequency of 30 Hz for two times for 1 min and stored at −80 °C. All the following analyses were performed in three biological replicates with three plants pooled per each replicate.

Biochemical analysis

Lipid hydroperoxides were quantified in leaf tissue by oxidation of a Fe(II) xylene orange complex as described by Frolov et al. (58), and the levels of TBA-reactive substances (expressed as malondialdehyde equivalents) were analyzed according to Velikova et al. (59) using ~25 mg of frozen ground plant material. The leaf tissue contents of ascorbic acid (Asc), DHA, and H2O2 were determined according to Frolov et al. (53). Quantification of reduced and oxidized glutathione relied on the method described by Bilova et al. (30). The contents of chlorophylls a and b were determined by spectrophotometric method of Arnon (60). For expression analysis of selected genes coding anti-oxidative and anti-glycative proteins (cytosolic ascorbate peroxidase cytosolic (APX1, At1g07890), cytosolic glutathione reductase (GRcyt, At3g24170), glyoxalase 1 (GLX1, At1g08110), and glyoxalase 2 (GLX2, At2g43430)), primers were designed by OligoPerfect™ Designer (supplemental Table S1-7) and purchased from Eurofins Genomics. The expression analysis was performed as described by Paudel et al. (53) using commercially available kits: NucleoSpin® RNA plus kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) for the total RNA isolation; maxima H minus first strand cDNA synthesis kit (Thermo Fisher Scientific, Darmstadt, Germany) for the cDNA synthe-
sis; and 5× QPCR Mix EvaGreen® (No ROX) kit (Bio&SELL, Feucht bei Nürnberg, Germany) for the RT-qPCR. As only well-established assays were employed here, all experiments (except RT-qPCR) relied on one analysis per each biological replicate. RT-qPCR experiments relied on 3–6 technical repetitions per each biological replicate.

Proteomic analysis

Isolation of proteins, removal of low molecular weight contaminants, and determination of glyoxalase 2 activity were performed according Bilova et al. (30). The protein contents were determined by the Bradford assay in a 96-well format and validated by SDS-PAGE, as described elsewhere (61). For the analysis of carboxylated proteins, anti-dinitrophenylhydrazine Western blotting was performed as described by Frolov and co-workers (53). For tryptic digestion, 2 aliquots of each purified extract, containing 100 μg of protein, were supplemented with 50 mM aq. ammonium acetate buffer to obtain a final volume of 90 μl and digested with trypsin by the procedure of Fedorova and co-workers (62). The digestion efficiency was verified by SDS-PAGE, as described elsewhere (33). For the analysis of the early glycation products, one of the digested 100-μg aliquots was subjected to boronic acid affinity chromatography (BAC) using a previously established procedure (30, 63). The BAC eluates and non-enriched digests were desalted by reversed-phase solid-phase extraction (64). The obtained SPE eluates were lyophilized overnight and reconstituted in acetonitrile containing 0.1% (v/v) formic acid (final acetonitrile concentration of 3% v/v) to obtain a peptide concentration of 7 mg/liter.

For the LC-MS analyses, 10 μl of each sample were injected in a nanoAcquity UPLC system controlled by MassLynx X.4.1 software and equipped with nanoAcquity UPLC™ Symmetry® trap (C18, 180 μm × 20 mm, 5 μm, 100 Å) and BEH130 separation (C18, 100 μm × 100 mm, 1.7 μm, 130 Å) columns (Waters GmbH, Eschborn, Germany). Glycated peptides were separated in linear gradient mode and detected on line by an LTQ-Orbitrap XL ETD mass spectrometer equipped with a nano-electrospray ionization source and controlled by Xcalibur 2.0.7 software (Thermo Fisher Scientific, Bremen, Germany) as described by Bilova et al. (30). Peptide sequence analysis and identification of glycated proteins relied on the database search against an A. thaliana database (Arabidopsis thaliana proteome, Uniprot, http://www.ebi.ac.uk/reference_proteomes, May 2014) using Sequest search engine. The positive hits were searched against a decoy database. The assignment of specific AGE modifications relied on characteristic m/z increments and appropriate search filters (XCorr ≥2.2 and 3.75 for doubly- and triply-charged quasi-molecular precursor ions, respectively, and false discovery rate of 0.01, see supplemental materials 1 and supplemental Table S1-8).

Label-free quantification of individual glycation sites was performed by integration of the corresponding peptide signals in specific (XICs, m/z ± 0.02) Orbitrap MS-only experiments at characteristic I_R using LCQUAN™ software (Thermo Fisher Scientific, Bremen, Germany). Based on our earlier validation study (60), one injection per each biological replicate was employed. The protein functions were annotated using MapMan software (Max-Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany). Glycation motifs included original protein sequence fragments of 25 amino acid residues prior to and after Arg- or Lys-modified sites and were investigated by sequence logo using the WebLogo application (65). The mass spectrometry proteomics data have been deposited on the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository (66) with the data set identifier PXD006434.

Metabolite profiling

Metabolite profiling relied on gas chromatography-mass spectrometry (GC-MS, one injection per biological replicate), performed with Agilent Technologies 6890 gas chromatograph (1 ml/min helium flow, splitless mode, injector temperature 250 °C) equipped with a 7683 autosampler (CTC Analytics, Zwingen, Switzerland) and coupled on-line to an electron ionization quadrupole mass spectrometer (EI-Q-MS, MSD 5973, Agilent Technologies, Böblingen, Germany), operated at an electron energy of 70 eV. For the analysis of primary metabolites, ~20 mg of ground plant material was extracted as described by Birkemeyer and co-workers (67); 110 μl of the extract was transferred to a new polypropylene tube; and the solvent was evaporated under reduced pressure. The residues were sequentially derivatized with methoxyamine hydrochloride in pyridine, and MSTFA prior to GC-EI-Q-MS analysis as described by Milkovska-Stamenova and co-workers (68). The metoxime-trimethylsilyl derivatives were annotated by characteristic t_R, I_R, and mass spectral similarity search against an in-house library (carbohydrates) or the NIST08 database (other metabolites). The t_R values were determined with n-alkane mixture (supplemental Table S1-2 and supplemental Fig. S1-1). To compare the I_R values of carbohydrates with those of authentic standards (supplemental Table S1-3), mixture of the latter as well as n-alkane mixture were analyzed directly prior to the samples (supplemental Figs. S1-1 and S1-2). Quantitation of metabolites was performed by integration of the corresponding extracted ion chromatograms (m/z ± 0.5 Da) for representative intense signals at specific t_R. Reactive α-dicarbonyl compounds, i.e. glyoxal (GO) and methylglyoxal (MGO), were derivatized by addition of O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine directly to plant extracts and quantified by a standard addition approach as described by Bilova et al. (30).

Statistical analysis

Based on quantitative data obtained, means ± S.D. were calculated, and significance of differences between them was tested with Student’s t test at p ≤ 0.05. To control false discovery rates, Benjamini-Hochberg correction of p values (69) was performed by using p.adjust function from R-package.

Modeling and calculations

Protein homology modeling of aging-specific glycated proteins (Fig. 10 and supplemental Table S-6) was automatically performed with YASARA (70). The resulting models were evaluated by YASARA, and if appropriate a final model was created by merging best folded fragments from different
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models followed by energy minimization. The quality of all models was checked for native folding by energy calculations with PROSA II (71) and for stereochemoical quality by PROCHECK (72). The models were manually inspected for the detection of the AGE modification sites by using the “molecular operating environment” program package MOE 2015.1001 (https://www.chemcomp.com/) (75).4

The domain localization of AGE sites in proteins relied on the search against InterPro (https://www.ebi.ac.uk/interpro/about.html),4 Pfam (http://pfam.xfam.org/),4 and SMART (http://smart.embl.de/) databases (49, 73, 74). The occurrence of individual amino acid residues in specific positions relative to the AGE modification sites was calculated by means of the WebLogo software tool using 25 amino acid residues N- and C-terminally from the glycation site as described by Crooks et al. (65). The FASTA files containing the sequences of selected proteins were constructed by means of the Retrieve/ID mapping tool (http://www.uniprot.org/uploadlists/).4

Author contributions—T. B. and A. F. designed the experiment, planned the entire work, and performed proteomic studies. D. B., G. P., and E. T. performed plant experiments and stress characterization and were involved in metabolomics studies. T. B. and C. B. designed and performed metabolic experiments. T. B., S. M., and G. S. performed processing of metabolomics data. S. M., T. B., R. S., A. F., and W. B. performed sequence and structure analysis. T. B., A. S. N., S. A., T. V., W. B., L. A. W., and A. F. contributed to data interpretation, critical discussion, and manuscript writing.

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