Chapter 12

Glycation of Plant Proteins under Environmental Stress — Methodological Approaches, Potential Mechanisms and Biological Role

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Abstract

Environmental stress is one of the major factors reducing crop productivity. Due to the oncoming climate changes, the effects of drought and high light on plants play an increasing role in modern agriculture. These changes are accompanied with a progressing contamination of soils with heavy metals. Independent of their nature, environmental alterations result in development of oxidative stress, i.e. increase of reactive oxygen species (ROS) contents, and metabolic adjustment, i.e. accumulation of soluble primary metabolites (amino acids and sugars). However, a simultaneous increase of ROS and sugar concentrations ultimately results in protein glycation, i.e. non-enzymatic interaction of reducing sugars or their degradation products (α-dicarbonyls) with proteins. The eventually resulting advanced glycation end-products (AGEs) are known to be toxic and pro-inflammatory in mammals. Recently, their presence was unambiguously demonstrated *in vivo* in stressed *Arabidopsis thaliana* plants. Currently, information on protein targets, modification sites therein, mediators and mechanisms of plant glycation are being intensively studied. In this chapter, we comprehensively review the methodological approaches for plant glycation research and discuss potential mechanisms of AGE formation under stress conditions. On the basis of these patterns and additional *in vitro* experiments, the pathways and mechanisms of plant glycation can be proposed.

Keywords: Advanced glycation end-products (AGEs), Ageing, Environmental stress, Glycation, Proteomics

1. Introduction

1.1. Environmental stress, ROS and protein glycation

1.1.1. Environmental stress and ROS formation

Environmental stress is one of the major factors reducing the productivity of crop plants all over the world [1]. Drought, high light, salinity and increased heavy metal soil contents, as well as extreme temperature, represent its important manifestations [2]. On the physiological level, extreme environmental conditions ultimately results in decrease in the CO_2 assimilation rate and in growth inhibition [3]. Simultaneous accumulation of reduced equivalents results in an overload of the chloroplast and mitochondrial electron transport chains and enhanced production of the reactive oxygen species (ROS), i.e. singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\cdot -}$), peroxide ion (O_2^{2-}), hydrogen peroxide (H_2O_2), various hydroperoxides and hydroxide radical ($OH^{\cdot}$) [4]. When ROS production overwhelms their detoxication, oxidative stress develops [5].

Thus, transfer of electrons to molecular oxygen (O_2) from ubisemiquinone in mitochondria and thylakoid membrane-bound primary electron acceptor of photosystem I (PSI) in chloroplasts yields $O_2^{\cdot -}$ (and, when further reduction occurs, O_2^{2-}), further converted to H_2O_2 by superoxide dismutase (SOD) activity (predominantly Mn- and CuZn-SOD in mitochondria and chloroplasts, respectively) [6,7]. The radical oxygen species can abstract protons from (bis-)allylic methylenes of polyunsaturated fatty acids (PUFAs) [8]. The subsequent capture of O_2 molecule by the resulting carbon-centered radical yields a peroxyl radical, that is able to initiate a chain reaction of lipid peroxidation [9]. The PUFAs can be directly attacked by the protonated form of $O_2^{\cdot -}$ ($HO_2^{\cdot}$) [10], thus the content of lipid hydroperoxides is one of the most reliable markers of oxidative stress.

The hydroperoxides can be easily involved in the Fenton reaction, i.e. transition metal ion-mediated reduction, yielding $OH^{\cdot}$, i.e. one of the most short-living and toxic ROS, directly and irreversibly modifying lipids, proteins and nucleic acids [11]. The metal ions oxidized during Fenton reaction are reduced *in vivo* by cellular antioxidants or $O_2^{\cdot -}$ (Haber-Weiss reaction), that considerably increases the production of $OH^{\cdot}$ [12]. As environmental stress is accompanied with a strong upregulation of mono- and oligosaccharides in all plant tissues [13], metal-catalyzed oxidation of sugars (so-called monosaccharide autoxidation) [14] also might be enhanced under the conditions of oxidative stress. The resulting products – hydroxycarbonyls and α-oxocarbonyls – are the potent protein modification agents and can induce essential changes in their structure and function [15]. These reactions, termed protein glycation, i.e. modification of proteins by carbonyl compounds (carbohydrates and α-oxocarbonyls), is believed to be an important factor in stress-related protein damage [16].

1.1.2. Protein glycation

In the first step of this process (usually termed "early glycation"), reducing sugars, aldoses and ketoses reversibly interact with amino groups resulting in the very labile N/O-acetal

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intermediates: aldoamines and ketoamines, respectively (Figure 1). These compounds easily condense yielding aldimines and ketoimines (Schiff bases), which undergo Amadori [17] and Heyns [18] rearrangements, variants of the acyloin shift. Amadori rearrangement involves proton transfer from C1 to C2 via the enol/enamine intermediate yielding *N*-substituted 1-amino-deoxy-ketoses, the Amadori products (Figure 1A). Similarly, in the case of ketoamines, in course of Heyns rearrangement [18], a proton migrates from C2 to C1 forming 2-amino-deoxyaldosyl adducts, often referred to as Heyns products (Figure 1B) [19]. Both Amadori and Heyns products are termed as "early glycation products", the first relatively stable intermediates of glycation [20].

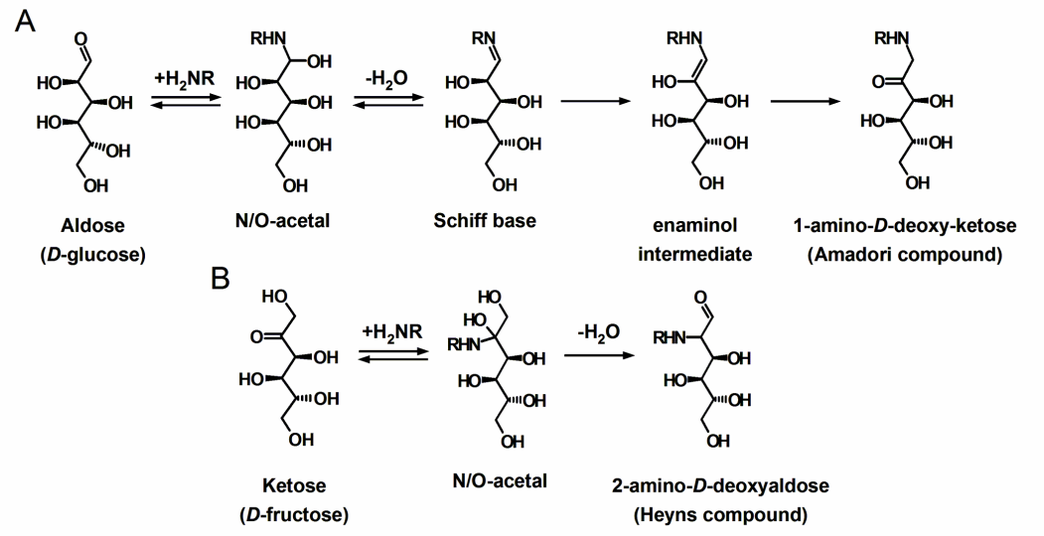

Figure 1. Formation of N/O-acetal and Schiff intermediates with subsequent Amadori rearrangement (A), and ketamine intermediates with subsequent Heyns rearrangement (B, not all intermediates shown).

These early glycation products, as well as free sugars, readily autoxidize (by the mechanisms similar to those described for free sugars) with formation of highly reactive α-dicarbonyl compounds (presumably glyoxal, methylglyoxal and various osones) – potent reactive intermediates of advanced glycation [21]. Depending on the structure of the carbohydrate moiety involved in this degradation, i.e. free sugars, or protein-bound early glycation products, two principle advanced glycation pathways, namely "oxidative glycosylation" and "glycoxidation", respectively, are distinguished [14,22,23]. The interaction of α-dicarbonyls with lysyl amino and arginyl guanidino side chain groups results in formation of so-called advanced glycation end-products (AGEs) – protein Maillard reaction compounds accumulating during thermal processing of food (Figure 2) [24], but also endogenously, e.g. under the conditions of persisting hyperglycemia.

1.1.3. Advanced Glycation End-products (AGEs)

AGEs represent a highly heterogenic group of compounds, varying greatly in their stability. Thus, the term "advanced glycation end-products" is, to high extent, conventional: some AGEs

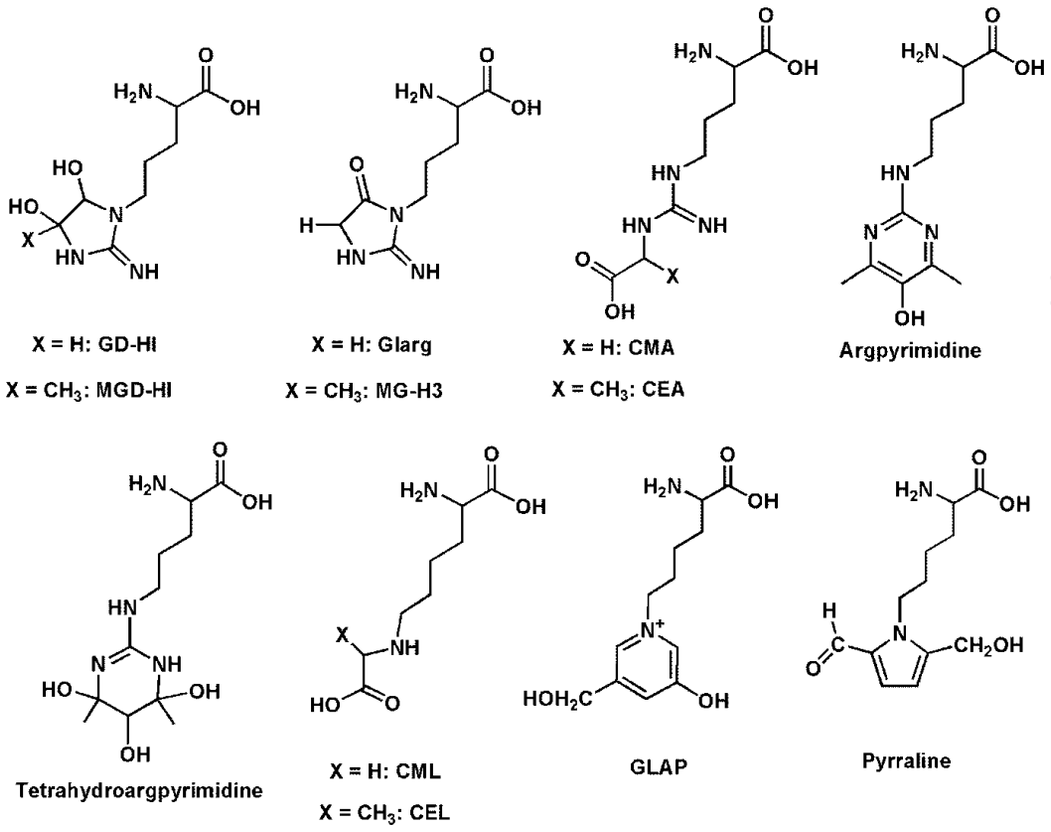

Figure 2. AGEs detected *in vivo*: GD-HI, glyoxal-derived dihydroxyimidazolidine; MGD-HI, methylglyoxal-derived dihydroxyimidazolidine; MG-H, methylglyoxal-derived hydroimidazolone; Glarg, glyoxal-derived hydroimidazolone; CML, N^{ε}-carboxymethyllysine; CEL, N^{ε}-carboxyethyllysine; GLAP, glyceraldehyde-derived pyridinium compound.

are still reactive and can be easily involved in further reactions [25]. In the past few decades, several AGEs were comprehensively characterized (Figure 2). Among lysine-derived modifications, N^{ε}-(carboxymethyl)lysine (CML) [26], N^{ε}-(carboxyethyl)lysine (CEL) [27], ε-(2-formyl-5-hydroxymethyl-pyrrolyl)-*L*-norleucine (pyrraline) [28] and glyceraldehyde-derived pyridinium compound (GLAP) [29] are the best-characterized. Not less attention was paid to the modifications of arginine. Thus, Schwarzenbolz and coworkers reported 1-(4-amino-4-carboxybutyl)2-imino-5-oxo-imidazolidine (Glarg) as a product of the reaction of arginine with glyoxal [30] and yielding N^{δ}-carboxyethylarginine (CMA) upon hydrolysis at 37°C [31]. Methylglyoxal was shown to form isomeric methylglyoxal-derived hydroimidazolones (MG-Hs) with N^{δ}-(5-methyl-4-oxo-5-hydroimidazolinone-2-yl)-*L*-ornithine (MG-H1) as the major isomer [32]. Hydrolysis of 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl)pentanoic acid (MG-H3) yields carboxyethyl-*L*-arginine (CEA) [33]. Sequential modification of arginine with two methylglyoxal molecules results in N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-*L*-ornithine (argpyrimidine, Argpyr) [34] and N^{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-*L*-ornithine (tetra-hydroargpyrimidine, TH-Argpyr) [35].

Upon their absorption in human intestine, AGEs interact with endothelial and macrophage pattern recognition receptors for AGEs (e.g. RAGEs) and trigger NF-κB-mediated expression

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of pro-inflammatory species (e.g. adhesion molecules, including vascular cell adhesion molecule-1 and intercellular adhesion molecule-1) [36] and foster the development of inflammatory diseases – e.g. atherosclerosis and type 2 diabetes mellitus [37]. AGEs of different chemical structure and origin, most often CML, pentosidine and hydroimidazolones, are known to be the ligands of RAGEs and to trigger inflammatory response [38].

Surprisingly, other reports showed mammalian serum and urinary concentrations of AGEs to be independent from dietary intake of thermally processed foods [39]. Moreover, the levels of CML and fluorescent AGEs in the plasma of vegetarian individuals were higher in comparison to those in the omnivorous individuals [40], even though the vegetarian diet had lower contents of lysine- and arginine-containing proteins. Remarkably, this effect was stronger in plasma of long-term vegetarians [41]. These facts indicate a high relevance of protein glycation (both early and advanced) in plants. Obviously, this explains the presence of multiple efficient anti-glycative enzymatic systems, like glyoxalase I and II [42], ribulosamine/erythrulosamine 3-kinase [43], acylamino acid-releasing enzyme [44].

Recently, Bechtold and co-workers reported an increase in the total contents of individual AGE classes upon the application of experimental environmental stress [16]. Thus, it is obvious that environmental changes are accompanied with enhanced generation of AGEs in plant tissues. In other words, due to the continuously altering growth conditions, AGEs might accumulate in plants during their life span, causing stress-related changes of the plant proteome and its physiological state. Important to note, that due to the dramatically different metabolic background (i.e. other patterns of carbohydrates, as well as high contents of potential antioxidants and carbonyl traps), pathways of glycation in plants may differ from those described in mammalians. However, no information about the proteins and biochemical pathways affected by such glycation reactions (i.e. its structural and functional patterns) was available until very recently. The most recent studies from our labs on the protein glycation patterns of model plants in the absence and presence of environmental stresses, as well as the impact of protein glycation in plant ageing, are added to this chapter.

2. Methodological approaches for the study of protein glycation in plants

2.1. Proteomics in plant glycation research

Recently, using liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based approach, Bechtold and co-workers estimated the total levels of individual AGE classes in *Arabidopsis thaliana* leaf tissues [16]. Their methodology relied on the tandem mass spectrometric (MS/MS) analysis of individual amino acids in tissue hydrolyzates by multiple reaction monitoring (MRM) [45]. Though this method provides high sensitivity and specificity, this strategy could not provide information about the identity and modification patterns of individual glycated proteins. However, this question can be addressed by the methods of LC-MS-based proteomics – the technique based on the analysis of protein enzymatic hydrolyzates

[46]. Indeed, in its classical implementation, unbiased LC-MS approach combines high separation efficiency of the high-performance or ultra-high-performance liquid chromatography (HPLC or UPLC, respectively) and outstanding resolution and mass accuracy of high-resolution mass spectrometry – Orbitrap- and quadrupole-time-of-flight (QqTOF)-MS [47,48]. It dramatically increases the analytical resolution (i.e. the number of analytes, annotated in one experiment) of the whole method. Moreover, due to the superior separation, low-abundant post-translationally modified peptides are less amenable to the ion suppression effects, accompanying electrospray ionization (ESI) [49]. Obviously, this analytical strategy seems to be the optimal for the study of such heterogenic and low-abundant set of modifications as AGEs.

2.2. Protein isolation and proteolysis

Depending on the target protein fraction (soluble or total), aqueous (aq.), extraction can be performed in the absence and presence of phenol, respectively [50]. In the first case, however, even the extracts of green parts contain high amounts of soluble metabolites that might inhibit the activity of proteases used for digestion. Such incompleteness of proteolysis can be observed as well, when proteins are extracted from plant parts rich in anti-nutritive (i.e. protease inhibiting or denaturing) phenolics like insoluble condensed tannins in seeds [51]. Therefore, the extracts can be purified by gel filtration chromatography and/or ultrafiltration using Centricon or Vivaspin centrifugation devices [52] prior to the determination of protein concentration. Alternatively, the proteins can be isolated by phenol extraction. In this case, phenolics contaminants can be removed by addition of 1–5% of soluble or insoluble polyvinylpyrrolidone [50]. While purified aqueous extracts can be easily digested by proteases in the presence of only deoxycholate as a denaturizing agent [53], the dried proteins isolated with phenol (containing also the fraction of hydrophobic membrane proteins) can be reconstituted only in the presence of both chaotropic compounds (urea, thiourea) and strong detergents. Conventional detergents, such as sodium dodecyl sulfate (SDS) or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), co-elute with proteolytic peptides and disturb ESI. This can be avoided by application of the detergents which do not impact protease activity and can be easily destroyed upon the digest. For example, *Rapi*Gest™ SF Surfactant (Waters Corporation, Milford, US) or Progenta Protea Biosciences, Inc., Morgantown, US) can be applied [54]. The detergents can be dissolved in the lysis buffer (7 mol/L urea and 2 mol/L in 50 mmol/L Tris-HCl, pH 7.5) and destroyed directly after proteolysis at pH 2 for 20–45 min (Figure 3).

The completeness of the digestion can be controlled by the SDS-polyacrylamide gel electrophoresis (PAGE). Performing SDS-PAGE additionally prior to proteolysis provides the opportunity to validate the Bradford assay results by relative quantification of total lane densities and, if necessary, normalize the results of the LC-MS-based label-free quantification. It is important to stress that the use of chaotropic agents, acid-labile detergents and strong acids for their cleavage ultimately require RP-based solid phase extraction (SPE) after completion of the digest and verification of its completeness by SDS-PAGE.

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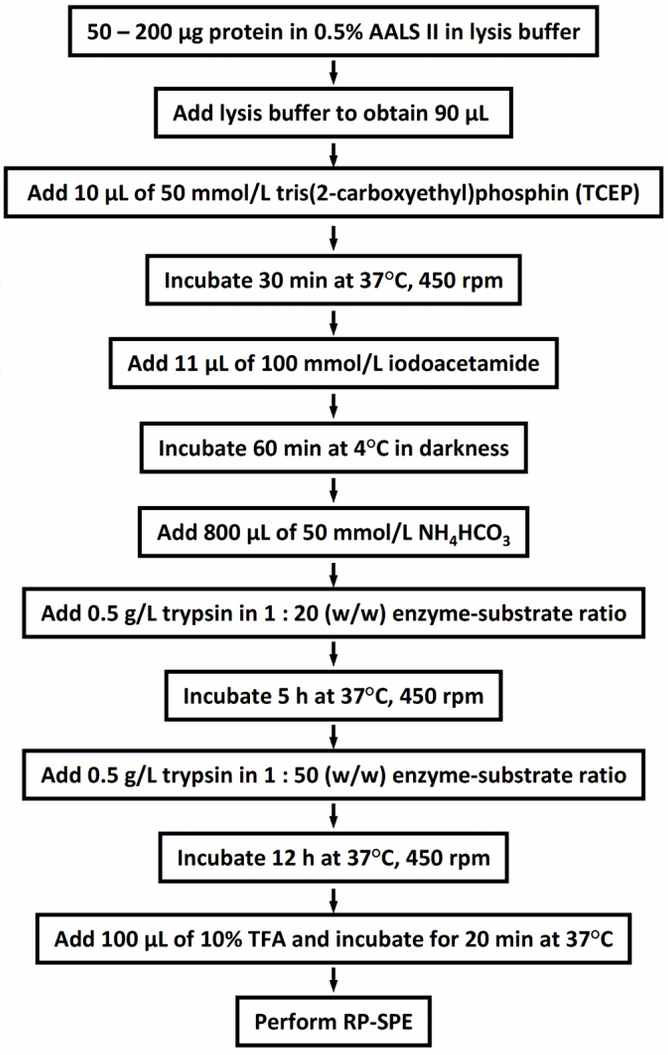

Figure 3. Protocol for tryptic digestion of the total plant protein obtained by phenol extraction. The procedure employed tryptic digestion in the presence of an acid-labile detergent ALLS II, required to ensure efficient solubilisation of membrane proteins.

2.3. LC-MS analysis of glycated peptide mixtures

The proteolytic digests are, typically, complex mixtures. Therefore, for successful detection and identification of their components (i.e. peptides), adequate chromatographic and mass separation techniques need to be applied. The selection of a strategy for LC-MS analysis (in terms of throughput, sensitivity, separation efficiency and reproducibility) depends on the aims of particular research. In the absolute majority of cases, LC-MS analyses rely on so-called data-dependent acquisition experiments (DDA) [55]. These complex experiments comprise survey MS scan (typically performed in Orbitrap- or TOF mass analyzer), and multiple dependent ones – MS/MS, relying on linear ion trap (LIT) or QqTOF analysis [56,57]. Modern instruments provide a possibility for introduction of the second dependent scan. Thus, hybrid LTQ-Orbitrap instruments support multi-step activation (MSA) experiments, comprising an additional MS/MS scan with a low-energy collision-induced dissociation (CID) [58]. The

modification-specific neutral losses, appearing under these conditions, trigger a high-energy dependent MS/MS scan providing rich structural information. In the past decade, these experiments were successfully applied to glycated tryptic digests using Amadori-specific losses of two and three waters, as well as additional formaldehyde molecule [59].

In the most straightforward way, DDA experiments are performed without additional analytical procedures preceding a RP-HPLC separation – so-called shotgun proteomics. However, as well as other PTMs, glycative and glycoxidative modifications are low abundant. Hence, their ionization might be suppressed by highly abundant species. To avoid this, early glycated tryptic peptides can be selectively enriched by boronic acid affinity chromatography (BAC) before LC-MS/MS analysis [60]. However, such enrichment is not possible for the whole fraction of advanced glycated peptides due to their structural heterogeneity. Therefore, these low-abundant species need to be directly detected in conventional data-dependent acquisition (DDA) experiments. As the DDA algorithm relies on the MS/MS analysis of the most intense signals in each time segment, this type of experiments suffers from so-called undersampling, i.e. missed fragmentation of low-abundant quasi-molecular ions [61]. Because of this reason, shotgun proteomics is not a desired strategy for the analysis of PTMs.

Thus, the number of co-eluting peptides in DDA analyses needs to be reduced to increase the coverage of the AGE-modified proteome. This can be addressed by three approaches: *(i)* introduction of enrichment or pre-fractionation step, *(ii)* gas phase fractionation (GPF) on the MS level and *(iii)* use of retention time-based exclusion lists. In all cases, for reliable identification of AGE-containing peptides, multiple DDA experiments are required. A special attention needs to be paid on the number of dependent MS/MS scans and the total duration of cycle. Thus, the number of MS/MS scans should not be too high, as the quasi-molecular ions of AGE peptides have relatively low intensities and require, therefore, longer fill times in LIT and accumulation time TOF. The cycle times typically need to be shorter, than in conventional shotgun DDA experiments, as the peak widths of low abundant peptides are smaller.

The generalized analytical strategy might comprise both qualitative and quantitative approaches, i.e. identification of glycated peptides in DDA experiments with their subsequent label-free quantification in additional full-scan MS experiments (Figure 4). For identification of glycation sites, early glycated peptides can be selectively enriched [60], while the analysis of AGE-containing species might rely on two-dimensional liquid chromatography (LC × LC) [62]. BAC is a well-established analytical tool to enrich Amadori and Heyns products from mammalian tissues (predominantly plasma) [56]. However, for application to the study of plant glycation, this method requires some optimization. Thus, the protein extract must be effectively washed prior to digestion (e.g. by ultrafiltration) to remove the co-extracted carbohydrate-related metabolites (mono-, oligosaccharides, sugar esters and glycosides of (poly)phenolics). Due to their *cis*-diol groups, these metabolites saturate the binding sites of the affinity column and reduce the peptide enrichment efficiency. Moreover, phenylpropanoids, as well as their sugar esters and glycosides, are well-retained on reversed phase [63] and might co-elute with peptides in course of RP-HPLC separation, leading to essential ion suppression during the ESI process and shift of peptide signals in the lower part on the instrument dynamic range. Thus, the analysis of early glycation proteins in total extracts that can not be efficiently ultrafiltrated is a challenging task.

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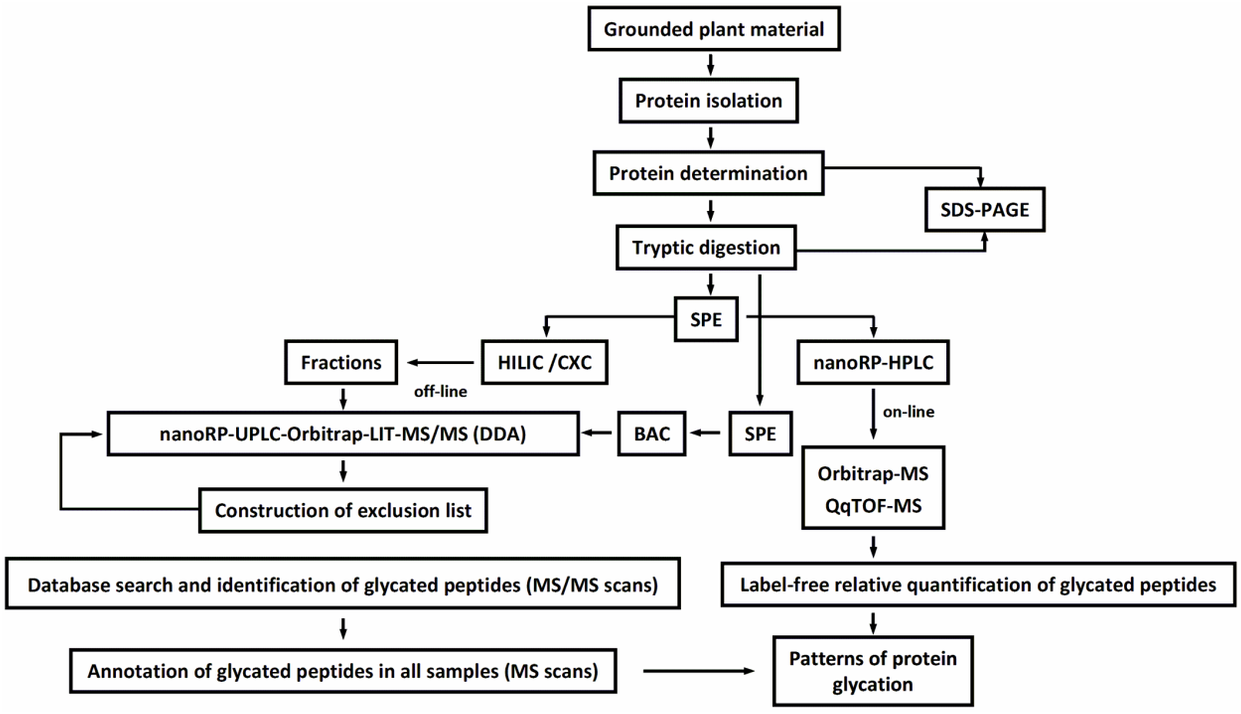

Figure 4. Experimental workflow for the analysis of glycated proteome. The analytical strategy comprises separation and MS/MS-based identification of modified peptides, as well as label-free quantification of corresponding glycated sites.

Pre-fractionation is usually introduced as an (relative to the RP) orthogonal separation procedure – most often cation exchange or hydrophilic interaction chromatography (HILIC) [62,64]. This approach essentially decreases sample complexity, facilitating, thereby, the fragmentation of low-abundant species. Similarly, fractionation can be applied on the MS level. Thus, simultaneously formed quasi-molecular ions (i.e. originating from the co-eluting species) can be fractioned by their *m*/*z* values in the quadrupole filter preceding LIT (GPF approach). In this case, however, repeated injections are necessary to cover the whole mass range that increases the overall analysis duration. For the most of applications, three GPF segments (*m*/*z* 400–600, 600–800 and 800–1500) are sufficient to significantly improve protein identification rates [57,64]. Additionally, based on the results of one DDA experiment, all unmodified peptides can be excluded from fragmentation in following ones on software level by generation of a corresponding "exclusion list" in the instrumental method [57]. However, the combination of these approaches is required for in-depth proteome analysis. The peptides can be identified by database search using SEQUEST or Mascot search engines.

Early glycated tryptic peptides can be annotated in high-resolution Orbitrap-MS experiments by the *m*/*z* value and charge of the corresponding quasi-molecular ion, and retention time in corresponding extracted ion chromatograms (XICs) as shown in Figure 5A [57]. Unambiguous identification of these peptides relies on characteristic series of b- and y-ions with consideration of the losses of three water molecules (pyrylium ions) and additional loss of formaldehyde (fulylium ions) [59] (Figure 5A). Identification and quantification of AGE-modified peptides are based on the same principle [65]. The only difference in comparison to the early glycation

species is the absence of the neutral losses accompanying parent and fragment ions. Additionally, the glycation state of peptides can be confirmed by characteristic fragments in the low *m*/*z* range [61,66]. This information can be further used for label-free quantification of individual AGE-modified peptides and, hence, specific glycation sites. The quantification typically relies on the integration of the annotated signals and qualitative comparisons of obtained peak areas. Thereby, the peak areas can be normalized to the signals of unmodified peptides in the same sample or in quality controls (QC). The peak integration can be performed by means of the vendor software packages – Xcalibur Quan Browser or LCquan.

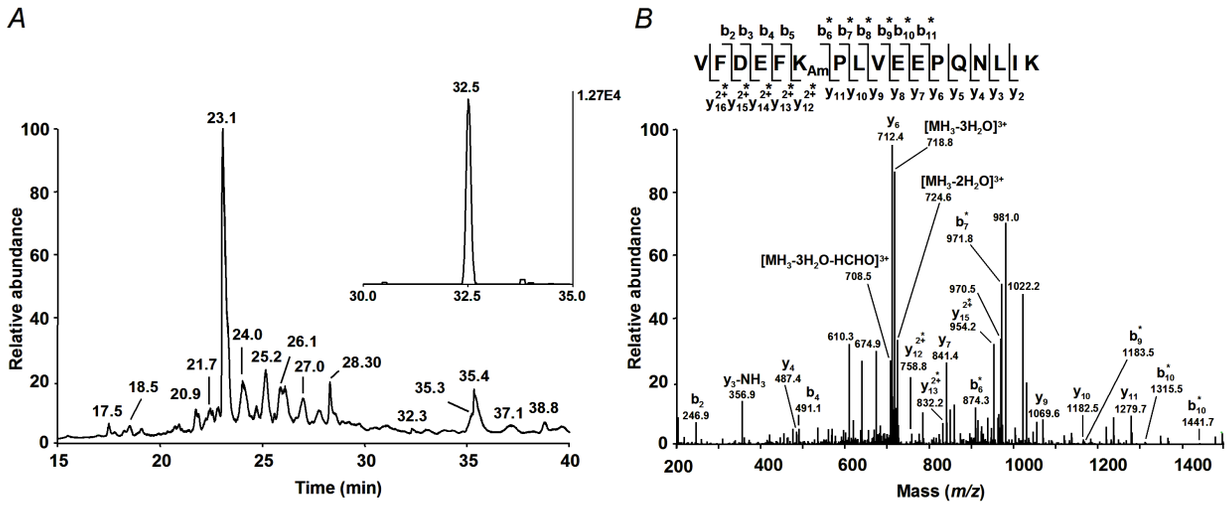

Figure 5. Annotation of Amadori peptides by nanoUPLC-ESI-LTQ-Orbitrap-MS/MS DDA experiments. Reversed phase ESI-Orbitrap-MS total ion chromatogram and the segment of the extracted ion chromatogram (insert) for *m*/*z* 736.39 ± 0.02 in the t_R range of 30–35 min (A) and an ESI-LTQ-MS/MS spectrum at *m*/*z* 736.4 corresponding to the $[M+3H]^{3+}$ ion of the peptide VFDEFK$_{Am}$PLVEEPQNLIK (B) acquired at 32.5 min of the same DDA experiment. Pyrylium b and y fragment ions are marked with asterisk [61].

The physiological role of glycation can be assessed by the system biology software tools. Thus, for the grouping of AGE-modified proteins by their functions, the mapping software MapMan (Max-Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany, http://mapman.gabipd.org) can be used. The functional annotation of proteins might give insight in biological effects of AGEs in plants and provide the material for future biological studies. Thus, the proteomic data can be complemented by the result of transcriptional analysis and determination of enzymatic activities. Afterwards, the functional role of glycation in respect of particular proteins can be confirmed by the experiments with corresponding mutants.

3. *In vivo* glycation of plant proteins

3.1. Plant protein glycation patterns

The possibility of plant protein glycation is considered since the beginning of the past decade, when Yamauchi and co-workers proposed the formation of AGEs as one of the possible

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mechanisms underlying inactivation of ribulose bisphosphate carboxylase/oxidase (RuBisCO) by high light [67]. Thereby, they proposed ascorbic acid as a possible precursor of AGEs. Indeed, this highly abundant compound in plant tissues can easily autoxidize and is recognized, therefore, as a potent glycation agent [68]. However, besides ascorbic acid, photosynthetically active leaf tissues contain high amounts of highly reactive pentoses, tetroses and trioses, as well as their phosphorylated forms, that might be even more reactive [69]. Probably, these sugars could be an important factor of light-dependent glycation. Recently, Bechtold and co-workers provided *in vivo* confirmation of this assumption: the authors found that tissue fructosyl lysine contents (determined by LC-MS/MS in exhaustive enzymatic protein hydrolyzates) are approximately four-fold higher in the day time in comparison to the dark period. It was not, however, the case for AGEs – just minor changes in their contents were observed during the day.

Implementation of the proteomic approach resulted in identification of proteins involved in glycation and exact modification sites therein. This strategy allowed identification of several hundreds of polypeptides containing early glycation moieties. Interestingly, the number of modification sites was higher in *Arabidopsis thaliana* in comparison to its close relative *Brassica napus*. Thus, glycation patterns might vary between species, although in both cases they are dominated by triose- and tetrose-derived products, accompanied with less abundant groups of pentose-modified sites, while hexose-derived modifications (typically the most representative in mammals) were less abundant.

Surprisingly, in plant proteins, the numbers of AGE-modified residues are essentially higher in comparison to the early glycated sites: approximately three- and seven-fold differences were observed for *A. thaliana* and *B. napus* proteome, respectively. It is the principle difference from glycation in mammals: though thousands of early glycated proteins were identified in human plasma and red blood cell membranes [64,70], only several dozens were proved to be AGE-modified [61,66]. Interestingly, the AGE modification sites in plant proteins are not accompanied with their early glycated counterparts, and are mostly originating from glyoxal and methylglyoxal. This situation differs drastically from the observations done with mammals. Indeed, several confirmed AGE sites in blood proteins (at least those representing the major plasma polypeptide human serum albumin, HSA) resembled the early glycated residues, indicating glycoxidation as an important pathway of AGE formation *in vivo*. The absence of such glycation sites in plant proteome clearly indicates the early glycation products as unlikely precursors of AGEs in plants. In this context, oxidative glycosylation rather than glycoxidation might be the predominant AGE formation pathway in plants. Remarkably, the number of early glycated lysyl residues was not only absolutely but also relatively (in comparison to the number of AGE-modified sites) lower in *B. napus*, than in *A. thaliana*. Most probably, it indicates higher activities of deglycation enzymes in the former plant.

It was shown in mammalians that the proteins controlling gene expression (e.g. transcription factors or the molecules involved in protein metabolism) can be the targets of glycation [71]. The same was demonstrated for plants. This might indicate the involvement of AGE formation in the regulation of gene expression on the levels of transcription and protein biosynthesis. This can be explained by the role of protein degradation in AGE metabolism and high

representation of arginyl residues in the transcription factors that makes these molecules highly amenable to interaction with α-dicarbonyls [71].

3.2. Protein glycation and environmental stress

3.2.1. Experimental models for the study of plant glycation

The main environmental stresses the plant can encounter in its habitat are high irradiation, contamination with heavy metals or metalloids and drought. It is necessary to take into account that drought is a general manifestation accompanying water deficit and is characteristic for the response to some other environmental factors, like high salt contents in soil or extremely low or high temperatures [72]. Obviously, for the study of any stress-specific response, selection of an appropriate model is of the principle importance. In this context, the researcher needs to be able to define all stress parameters by the selected experimental setup. This can be relatively easily achieved for a high light stress by using a phytotron equipped with the lamps providing required light intensity and complete climate control. In this case, a soil model can be applied (Figure 6) [73]. However, this approach does not provide the conditions equal for all plants, when a heavy metal stress is applied. That is why, growth of plants in aqueous (aq.) culture with a subsequent addition of a heavy metal salt to a growth medium seems to be a more adequate solution [74] (Figure 6).

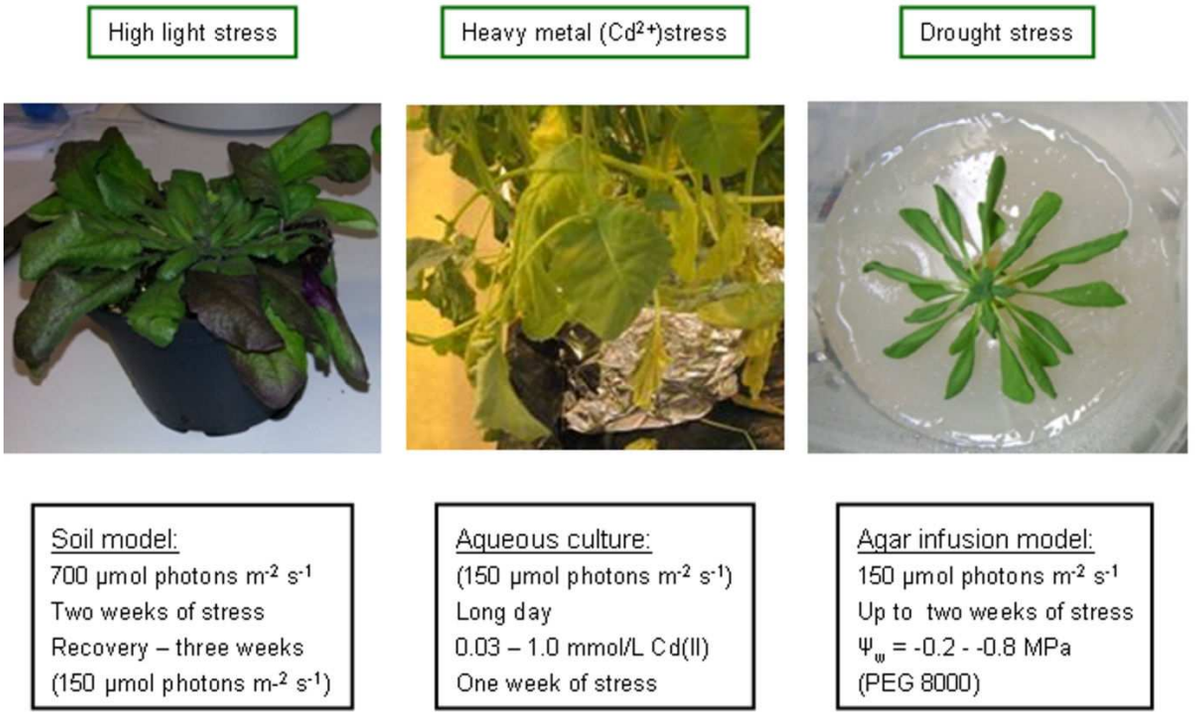

Figure 6. Experimental models of environmental stress.

As far as the drought stress is concerned, the water deficit can be modeled both in soil and in aqueous systems. According to Boyer [75], this water deficit can be expressed as a decrease of

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water potential (Ψ_w), i.e., as a difference of water potentials in a solution and pure water divided by partial molar volume of water. However, the soil-based approaches do not allow experimental definition of Ψ_w, as in this case it depends from the water absorption by plant. In aqueous culture, osmotically active substances, like commonly used sorbitol or polyethylene glycol (PEG), can affect the function of root epidermis [72]. Because of this, agar-based PEG infusion model, introduced in the past decade by van der Weele and co-workers [76], and additionally optimized to mature plants (Figure 6) seems to be more suited for this purpose. The confirmation of the stress development might rely on the determination of native leaf fluorescence [77], hydrogen peroxide contents [16] and some further parameters.

3.2.2. Stress-related glycation of plant proteins

Light is one of the most essential factors determining plant growth, development and survival [78]. However, a continuous exposure of plants to the high doses of sun irradiation might exceed the capacity of the mesophyll photosynthetic apparatus and trigger development of the oxidative stress [7]. In the context of the protein Maillard chemistry, discussed above, it is logical to assume that enhanced ROS and monosaccharide production would lead to the increase of AGE formation in plant green tissues. As was proposed earlier, such modifications of the RuBisCO polypeptide chains might impact inactivation of the enzyme with high light [67]. Similar mechanisms might accompany the development of drought and metal stress. Moreover, the tissue metal-scavenging mechanisms include activation of the enzyme phytochelatinsynthase, requiring GSH as a substrate [79]. Hence, glyoxalase system, critical for detoxication of methylglyoxal (MGO) and utilizing GSH as a substrate as well [80], can not perform efficient scavenging of this dicarbonyl under stress conditions, which might stimulate enhanced glycation by MGO in the proteins of metal-treated plants.

The effect of stress on the formation of AGEs differs on the qualitative and quantitative levels. Thus, in most cases, relatively low number of stress-specific glycation sites can be detected in the proteins of stressed plants, and such sites are representing mostly the molecules involved in transcription and protein degradation (i.e. those known to be upregulated under stress conditions) [81]. This could be explained by the activation of some unknown enzymatic systems eliminating either AGE precursors, or AGE-modified proteins, or their early glycated precursors, i.e. Amadori and Heyns compounds. Indeed, activation of the glyoxalase system (comprising enzymes glyoxalase I and II) is well documented during environmental stress [82]. Additionally, although in mammals advanced glycation decreases the rates of proteolysis, in plants it can be not the case, that was confirmed by *in vitro* experiments [67]. Thus, acylamino acid-releasing enzyme might impact in plant protein degradation pathways [44]. However, the pathways, involved in enhanced degradation of glycated proteins in plants still need to be studied. Finally, Amadori and Heyns products can be degraded via phosphorylation pathway [43]. Besides, the possibility of the further reactions of AGEs to form new unknown products also needs to be considered [83].

Compared to the qualitative alterations, the quantitative stress-related changes in glycated proteome are much more pronounced. Thus, several AGE classes were at least two-fold increased even after short application of light stress [16]. This tendency could be followed on the level of individual glycation sites. However, changes in representation of a certain AGE moiety need to be verified on the level of the abundance of corresponding protein, i.e. the abundance of specific modification sites need to be considered together with the data on gene expression on the RNA and protein levels. Moreover, the changes in protein degradation rates need to be taken into account.

As was demonstrated in the pioneer study of Bechtold and co-workers, stress-related upregulation of early glycation products is much stronger in comparison to AGEs [16], which was confirmed on the level of individual glycation sites. Thus, stress conditions lead to the considerable increase of the contents of Amadori and Heyns adducts, while the response on the level of advanced glycation is much lower. Moreover, stress-specific AGEs are dominated by α-dicarbonyl-derived products, and only few could originate from Amadori or Heyns products. The negligible role of early glycation products in AGE formation additionally supports the existence of powerful Amadori/Heyns product-degrading enzymatic mechanisms in plant tissues. Moreover, it might indicate the presence of a potent stress-inducible anti-glycation system scavenging or/and reducing α-dicarbonyls. This assumption can be supported by the absence of changes in the carbonylated proteome, as well as glyoxal and methylglyoxal contents throughout the stress development. Thus, scavenging of these advanced glycation intermediates by the amino functioned metabolites might be the most probable scenario.

3.3. *In vitro* modeling of plant glycation reactions

Due to their high photosynthetic activity, green parts of plants are characterized with high contents of carbohydrates. Thus, for *B. napus* leaf tissue, these contents were more than 1 μmol/g f.w. for the major sugars (glucose, fructose, sucrose, Asc and DHA) and were in the range of 10–60 nmol/g f.w. for such compounds as glucose-6-phosphate, ribose and arabinose that are known to highly-reactive [84,85]. Hence, metabolic background of plant glycation differs essentially from that reported for mammals. Thus, animal tissues are rich in glucose (that is known to be one of the weakest glycation agents), while the contents of more reactive sugars are negligible [86]. This strong prevalence of one potential glycation agents dramatically affects the mammalian glycation patterns, which are strongly dominated by the protein fructosamines, i.e. glucose-derived Amadori compounds [64]. In contrast, photosynthetically active tissues are rich in trioses, tetroses and pentoses as well, that might affect the early glycation patterns. Moreover, these highly reactive carbohydrates might be much more susceptible to autoxidation, i.e. represent potent precursors of α-dicarbonyls and, hence, AGEs. Obviously, the ability of certain sugars to act as a glycation agent depends not only on its tissue content but also on its ability to react with proteins. However, the reactivities of individual plant carbohydrates towards protein lysyl and arginyl residues, as well as their glycation potential, are still unknown.

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To address this question, *in vitro* glycation models based on synthetic peptides were established recently [24,83]. Ideally, such model peptides are N-terminally protected and contain only one residue (lysine or arginine, typically in mid position) susceptible to glycation. Essential advantage of such systems is their relative simplicity: the products can be separated and analyzed by tandem mass spectrometry (MS^n). Evaluation of peptide tandem mass spectra can be complemented by adequate methods of carbohydrate and α-dicarbonyl analysis [87,88]. These peptide models can be potentially applied to the assessment of reactivity of individual glycation agents (i.e. sugars and dicarbonyls) and determination of their glycation potential. Such analyses might rely on integration of selected XICs (characteristic for individual glycation products) at specific retention times. This would give a possibility for screening of plant sugars for their ability to form certain Amadori/Heyns compounds and AGEs. This would essentially impact in understanding of plant glycation pathways.

4. Conclusions

To conclude, protein glycation is a common post-translational modification in plants. Despite this, essential differences in comparison to mammalian glycation patterns were observed. Thus, glycation patterns are strongly dominated by AGEs, while the number of Amadori-modified lysyl residues is at least one order of magnitude lower compared to human plasma proteome. Moreover, individual AGE-modified sites are not represented by their Amadori/Heyns counterparts. It indicates autoxidation of free sugars rather than glycoxidation (i.e. AGE formation from early glycated products – one of the main glycation mechanisms in mammalians) as the major pathway of advanced glycation in plants. Environmental stress considerably affects glycation patterns, mostly on the quantitative level. However, due to the high heterogeneity of potential plant glycation agents, a high variability of glycation pathways and mechanisms can be expected. To clarify these pathways, simple *in vitro* models based on synthetic peptides can be used.

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