Protein glycation in plants – an under-researched field with much still to discover

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Abstract: Recent research has identified glycation as a non-enzymatic post-translational modification of proteins in plants. Protein glycation has a potential contributory role to functional impairment of the plant proteome. Reducing sugars with a free aldehyde or ketone group such as glucose, fructose and galactose, react with N-terminal and lysine side chain amino groups of proteins. A common early-stage glycation adduct formed from glucose is N_{ε}-fructosyl-lysine (FL). Saccharide-derived reactive dicarbonyls are arginine residue-directed glycating agents, forming advanced glycation endproducts (AGEs). A dominant dicarbonyl is methylglyoxal - formed mainly by trace-level degradation of triosephosphates, including Calvin cycle of photosynthesis. Methylglyoxal forms the major quantitative AGE, hydroimidazolone MG-H1. The concentrations of glucose and methylglyoxal in plants change with developmental stage, senescence, light and dark cycles and also likely with biotic and abiotic stresses. Proteomics analysis indicates there is enrichment of amino acid residue targets of glycation, arginine and lysine residues, in predicted functional sites of the plant proteome, suggesting susceptibility of proteins to functional inactivation by glycation. In this review, we give a brief introduction to glycation, glycating agents and glycation adducts in plants. We consider dicarbonyl stress, functional vulnerability of the plant proteome to arginine-directed glycation and the likely role of methylglyoxal-mediated glycation in activation of the unfolded protein response in plants. The latter is linked to the recent suggestion of involvement of protein glycation in sugar signalling in plant metabolism. Overexpression of glyoxalase 1, which suppresses glycation by methylglyoxal and glyoxal, produced plants resistant to high salinity, drought, extreme temperature and other stresses. Further research to decrease protein glycation in plants may lead to improved plant growth and assist breeding of plant varieties resistant to environmental stress and senescence – including plants of commercial ornamental and crop cultivation values.

Keywords: glycation, advanced glycation end products (AGEs); methylglyoxal; glyoxalase, dicarbonyl stress; unfolded protein response; *Arabidopsis*; *Brassica*; crops.

Abbreviations: AGEs, advanced glycation endproducts; AKR, aldoketoreductase; CEL, N_ε-(1-carboxyethyl)lysine CMA, N_ω-carboxymethylarginine; CML, N_ε-carboxymethyl-lysine; 3-DG, 3-deoxyglucosone; DHAP, dihydroxyacetonephosphate; ER, endoplasmic reticulum; FL, N_ε-fructosyl-lysine; GA3P, glyceraldehyde-3-phosphate; G-H1, glyoxal-derived hydroimidazolone, N_δ-(5-hydro-4-imidazolon-2-yl)ornithine; Glo1, glyoxalase 1; Glo2, glyoxalase 2; G6P, glucose-6-phosphate; GSH, reduced glutathione; HA, hemithioacetal; HSA, human serum albumin; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MG, methylglyoxal; MG-H1, methylglyoxal-derived hydroimidazolone, N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N^ε-lysino)-4-methyl-imidazolium; OsAKR1, aldoketo reductase isoform-1 of *Oryza sativa*; RBD, receptor binding domain; R5P, ribose-5-phosphate; SLG, S-D-lactoylglutathione; UPR, unfolded protein response.

1. Protein glycation in plants: three papers setting the scene.

In 2009, we published the first report on the steady-state levels of protein oxidation, nitration and glycation adducts in cytosolic protein extracts from leaves of *Arabidopsis thaliana* (thale cress) [1]. The cytosolic protein contents of the early-stage glycation adduct, N_{ε}-fructosyl-lysine (FL), and 8 advanced glycation endproducts (AGEs) were presented. This highlighted an aspect of the proteome of higher plants that is intuitive but had been little investigated: proteins are glycated in plants. In 2014, Takagi *et al.* showed that methylglyoxal (MG) - precursor of the major quantitative AGE, hydroimidazolone MG-H1 - is formed during photosynthesis in chloroplasts isolated from leaves of spinach [2]. In 2017, Bilova *et al*. published a proteomics study identifying proteins modified by AGEs in *A. thaliana* [3]. These studies revealed that higher plants produce glycating agents as a part of their vital photosynthetic metabolism and the proteome of plants is continually subjected to glycation forming early-stage glycation adducts and AGEs [4]. Further research in this area is, therefore, important to improve understanding of plant growth, resistance to environmental stress and senescence – including varieties of commercial importance for ornamental and crop production. Below in this review, we give a brief introduction to glycation, glycating agents and glycation adducts, and, following recent development in mammalian glycation, we consider dicarbonyl stress, the susceptibility of the plant proteome to functional inactivation by arginine-directed glycation and the role of glycation in activation of the unfolded protein response (UPR) in plants [5, 6]. The latter is likely linked to the recent suggestion of the involvement of protein glycation in sugar signalling in plant metabolism [7]. We limit our coverage to higher plants.

2. Glycation: the Maillard reaction.

Protein glycation is the non-enzymatic reaction of simple reducing sugars and related saccharide derivatives with proteins in a complex series of sequential and parallel pathways called the Maillard reaction [8]. Reducing sugars have an aldehyde or ketone group by which reactions occur with the protein substrate, typically on N-terminal and lysine side chain amino groups. In higher plants, examples of simple reducing sugars involved in protein glycation are: glucose, fructose and galactose. Glucose reacts with N-terminal amino groups and lysine residue side chain amino groups of proteins to form an initial Schiff's base which undergoes an Amadori rearrangement to form fructosamines: N_{α}-1-deoxyfructosyl N-terminal amino groups and N_{ε}-fructosyl-lysine (FL). The reactivity of reducing sugars with N-terminal amino groups is usually faster than with lysine side chain amino groups because of the lower pK_a of the former but lysine side chains are present at higher concentration than N-termini in the plant proteome. Some lysine side chain amino groups may also be activated towards glycation by interaction with neighboring cationic lysine and arginine residues, decreasing the pK_a of the lysine side chain amino group target [9]. Sucrose, a non-reducing sugar, is the main vehicle for sugar transport in plants. This fact greatly decreases the risk of glycation of the plant proteome by sugar in transit in the plant body (roots, stems and leaves) (44).

Common saccharide derivatives studied in glycation reactions are phosphorylated glycolytic intermediates, such as glucose-6-phosphate (G6P) and ribose-5-phosphate (R5P) – the latter an intermediate of the pentosephosphate pathway and Calvin cycle of photosynthesis in plants [10]. They react with and modify N-terminal and lysine side chain amino groups of proteins. There are also reactive dicarbonyl saccharide derivatives – such as glyoxal, MG and 3-deoxyglucosone (3-DG) [11]. Dicarbonyl metabolites are arginine-directed glycating agents, forming predominantly hydroimidazolone derivatives such as methylglyoxal-derived hydroimidazolone MG-H1 and related structural isomers, and analogous hydroimidazolones from glyoxal and 3-DG [12, 13]. For reaction of glyoxal with arginine, the ring-opened

rearrangement of the initial dihydroxyimidazolidine to N_ω-carboxymethyl-arginine (CMA) is favored [14]. Glyoxal reacts with lysine residues to form the AGE, N_ε-carboxymethyl-lysine (CML). CML is mainly formed by the oxidative degradation of FL [15], with formation also by glycation of lysine residues by ascorbic acid [16]. Glyoxal is formed in lipid peroxidation and the slow oxidative degradation of monosaccharides and proteins glycated by glucose [11]. MG is mainly formed by trace-level degradation of triosephosphates, glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetonephosphate (DHAP) [17]. GA3P is an intermediate in photosynthesis and GA3P and DHAP are intermediates in glycolysis, gluconeogenesis and glyceroneogenesis in lipid synthesis. 3-DG is formed by the enzymatic and non-enzymatic degradation of proteins glycated by glucose [11, 18].

Glycating agents and glycation adduct residues in proteins have been detected in plants. Glycation has been studied in plants of both research interest and commercial ornamental and crop relevance [1, 3, 19-24]. Levels of glycating agents and glycation adduct residues in protein vary with light and dark cycles, stage of development and environmental stresses [1, 24]. The molecular structures, metabolic source and likely functional significance of glycation adducts in plants is summarized in Table 1.

There are also enzymes of the anti-glycation defense which suppress protein glycation in plant tissues: the glyoxalase system which metabolizes glyoxal and MG and thereby suppresses the formation of related AGEs [25], aldoketo reductases (AKRs) that also metabolize glyoxal, MG and likely 3-DG [26], and ribulosamine/erythrulosamine 3-kinase - a putative enzyme of glycated protein repair which deglycates proteins glycated by R5P [10]. There is also acylamino acid-releasing enzyme which degrades glycated proteins [27].

Glycation adducts in plant proteins are formed by the slow *in situ* rate of protein glycation. They are removed by degradation of glycated proteins by cellular proteolysis. Some glycation adducts are repaired by deglycation enzymes [10, 27] and also by slow

spontaneous reversal of the protein glycation where glycation adducts have moderate stability [12, 13, 28]. The levels of glycation adducts measured, therefore, are the steady-state levels maintained by these conflicting processes. Steady-state levels of protein glycation adducts are influenced by light, stage of development, season, environmental stresses, nutrients and other factors influential on plant metabolism [1] (Table 2). Protein glycation has been implicated in the deterioration of plants seeds in storage [29]. The presence of enzymes in plants that suppress protein glycation and repair glycated proteins [25, 30] - the enzymatic defence system against glycation [31], suggests glycation of the plant proteome poses a threat to plant physiology and growth.

3. Protein glycation in *Arabidopsis thaliana.*

Studies of protein glycation in *A. thaliana* under normal growth and stress conditions are summarized in Table 2. Protein glycation was studied in *A. thaliana* by assaying protein glycation adducts in cytosolic protein extracts, using the reference method of liquid chromatography-tandem mass spectrometry (LC-MS/MS). This involves prior exhaustive enzymatic hydrolysis of proteins and quantitation of glycation adducts, glycated amino acids, by stable isotopic dilution analysis LC-MS/MS [13, 32, 33]. Under basal conditions, the mean extent of protein modification by major glycation adducts was: FL residues, 26%; MG-H1 residues, 4%; and CML residues, 3% [1].

There was a relatively high content of FL residues in plant protein on entering the daylight period – *ca.* 3 mmol/mol lys. This glycation adduct increased during and beyond the daylight period when photosynthesis leads to a 10-fold increase in glucose concentration of the leaf tissue [34] (Table 2). Increase of FL residue content reflects this increased exposure to glucose. CML residue content of plant protein was relatively high, 0.35 – 0.71 mmol/mol lys, and showed a similar trend to change in FL residue content. This may be due to the high content

of FL residues and ascorbic acid [35] - precursors of CML residue formation [15, 16]. Excess light stress also increased, CML and G-H1 residue contents of total leaf protein by *ca*. 2-fold. CML and G-H1 adduct residues are formed by glycation of proteins with glyoxal, which may relate to periods of increased lipid peroxidation and formation of glyoxal [36].

MG-derived MG-H1 residues were the AGE of highest content in the *A. thaliana* proteome at *ca.* 2 mmol/mol arg [1]. Other MG-derived AGE residue contents, N_{ε}-(1-carboxyethyl)lysine (CEL) and methylglyoxal-derived lysine dimer MOLD, were *ca.* 10-fold and 400-fold lower than MG-H1. All the AGE residues, except for CML, tended to show oscillatory diurnal behavior where maxima of residue contents occurring in the middle of the light and dark periods and lower levels of AGE residue contents occurring between these times [1].

In recent studies, the boronate affinity enrichment method was used to identify proteins in *A. thaliana* modified by FL residues, with protein identification by high mass resolution Orbitrap mass spectrometry proteomics. One hundred and twelve glycated proteins were identified [22]. FL modification and location of the glycation site in retained proteins was detected by mass spectrometric detection and fragmentation of peptides in tryptic digests using high sensitivity mass proteomics; the mass increment on lysine residues indicating presence of an FL residue is +162 Da. In this approach, however, there is typically a mean peptide sequence coverage of *ca.* 25% and, therefore, some glycation adducts are missed [37]. CML, CMA, G-H1 and MG-H1 residues were also detected in the retained proteins – although these adducts do not bind to boronate affinity columns [12, 13, 38]. The core group of 112 glycated proteins included chloroplast ATP synthase (β-subunit) and phosphoglycerate kinase; 90% of glycated proteins were of chloroplast origin [22]. The abundances of most of these glycated proteins were similar in experiments investigating the effects of light and heat, light and drought stresses. The number of glycated proteins changing in abundance in different stress conditions

were: light stress, 2; heat stress, 1; diurnal variation, 8; and drought conditions, 17 [22] (Table 2).

Proteins modified by early-stage glycation adducts and AGEs was examined in a proteomics study. AGE residues were detected at 96 different sites in 71 proteins, with age-dependent changes. Unique age-related proteins modified by AGEs (AGE, sequence location) in 9- and 12-week-old plants and pathways involved were: ß-carbonic anhydrase-2, chloroplastic (CMA, R202) and ACT domain containing protein ACR9 (MG-H1, R395) – involved in amino acid metabolism; putative fucosyl transferase-7 (CML, K393 and K394) – involved in cell wall biosynthesis; tetratricopeptide repeat-like superfamily protein (CEL, R83; MG-H1, R86) – involved in the oxidative stress response; and CEL – found in two uncharacterized proteins. Homology modeling revealed glutamyl and aspartyl residues in close proximity (less than 0.5 nm) to these sites in three aging-specific and eight differentially glycated proteins, four of which were modified in catalytic domains [3]. Protein domains of plant proteins susceptible to glycation have not been widely studied. In mammalian proteins, domains susceptible to glycation by MG were: tailless complex polypeptide-1 (TCP-1) and GroEL protein domains of chaperonins, 14-3-3 domain, α/ß subunits of the proteasome, class I and class II aminoacyl-tRNA synthetases, actin and Rossmann-like α/ß/α sandwich fold [6]. Similar domains in plant proteins may be susceptible to glycation by MG.

A comprehensive study of the changes in proteins modified by AGEs of *A. thaliana* in osmotic stress was reported [19]. Plants were grown from seed and at 6 weeks then transferred to new growth medium with and without polyethylenglycol-8000. This treatment is an experimental model of drought stress, inducing accumulation of osmolytes, amino acids and carbohydrates. After application of this osmotic stress for 3 days, changes in 31 stress-specific and 12 differentially AGE-modified proteins reflecting AGEs on 56 different sites was found

[19]. Monosaccharide autoxidation [39] was proposed as the main stress-related glycation mechanism, and glyoxal as the major glycation agent in plants subjected to drought [19].

4. Dicarbonyl stress in plants

Dicarbonyl stress is the abnormal accumulation of reactive dicarbonyl metabolites leading to increased protein glycation. It is linked to cell and tissue dysfunction, aging and disease [5]. MG is a key dicarbonyl metabolite contributing to dicarbonyl stress in plants, unavoidably formed by the non-enzymatic degradation of triosephosphates. It is formed at relatively high flux compared to other dicarbonyl metabolites, *ca.* 0.1% of glucose metabolism, and has high reactivity with proteins [5]. It is precursor of the major quantitative AGE in plant proteins, MG-H1 [24, 40].

We determined the content of glyoxal, MG and 3-DG of leaves of *Brassica oleracea* plants by the reference analytical method of stable isotopic dilution analysis LC-MS/MS [24]. We studied leaf dicarbonyl content at 3 stages of development (days post-seeding): cotyledons (6 days), first fully developed mature leaves (30 days) and mature plant (65 days). The glyoxal content of cotyledons was *ca.* 0.4 nmol/g fresh weight (*ca.* 0.4 µM); and it was similar at 30 days but increased 2-fold at 65 days. The MG content of cotyledons was *ca.* 3 nmol/g fresh weight (*ca.* 3 µM); and it was similar at 30 days but increased *ca.* 33% at 65 days. The 3-DG content of cotyledons was *ca.* 8 nmol/g fresh weight (*ca.* 0.8 µM); it increased to *ca.* 2-fold at 30 days and then decreased to levels similar to the cotyledon stage at 65 days (Table 3). Markedly higher estimates of MG content of plant tissue published previously of *ca*. 50 nmol/g fresh weight [41] were likely due to triosephosphate degradation to MG during pre-analytic processing [42]. Mathematical metabolic modeling of *in situ* glycation in physiological tissues predicts steady-state cellular concentrations of MG as 1 – 4 µM [24] The reactivity towards protein glycation of glyoxal, MG and 3-DG, compared to

that of glucose, is *ca.* 5,000, 20,000 and 200 higher. So, MG is expected to be the major dicarbonyl glycating agent in leaves of *B. oleracea*. The mature 65-day *B. oleracea* plant appears to be suffering dicarbonyl stress. The increase and later decrease of 3-DG content may reflect mobilization of glucose metabolism, declining in later stages of maturity; *cf.* measurements of glucose and fructose with plant development [43]. siRNA silencing of Glo1 and accumulation of MG in other species produced an accelerated aging phenotype whereas overexpression Glo1 increased longevity and produced resistance to metabolic dysfunction in ageing [44, 45]. Therefore, increased MG glycation may impair plant proteome integrity and vitality in older plants and provide a cue for senescence.

Prolonged use of ammonium salts as the sole nitrogen source for plants may result in physiological and morphological disorders leading to decreased plant growth. This is a worldwide problem, constraining crop production. It is a common example of abiotic stress [46]. The effect of this on dicarbonyl stress in *A. thaliana* was investigated. Changes in activities of glycolytic enzymes increased the formation and concentration of MG. Excessive accumulation of MG, dicarbonyl stress, produced increased MG-derived AGEs. Dicarbonyl stress may contribute to ammonium toxicity symptoms in *A. thaliana* and ammonium salt impairment of crop plant growth [47].

5. Enzymatic defence against glycation – glyoxalase system and aldoketo reductases.

The glyoxalase pathway catalyses the conversion of MG to D-lactate. It is a two-step, enzymatic pathway: glyoxalase 1 (Glo1) catalyses the conversion of the hemithioacetal formed non-enzymatically from MG and reduced glutathione (GSH) to S-D-lactoyl-glutathione (SLG); and glyoxalase 2 (Glo2) catalyses the hydrolysis of SLG to D-lactate, reforming the GSH consumed in the Glo1-catalysed step [48-50] (Figure 1). There is a further protein called glyoxalase-3 (Glo3) [51] but concern remains on its functional

attribution as a glyoxalase involved in MG metabolism physiologically due to its low catalytic efficiency. Catalytic efficiency is defined by the specificity constant, k_{cat}/K_M [52]. k_{cat}/K_M values for *A. thaliana* Glo1 (isoform 2, accounting for >99% Glo1 activity) and Glo3 are 1.1 x 10^{10} $min^{-1}M^{-1}$ and 3.4 × 10^6 $min^{-1}M^{-1}$, respectively [51, 53]; *ca.* 3,100-fold higher for Glo1 than Glo3. Proteomic abundances of Glo1 and Glo3 in *A. thaliana* were 1140 and 300 ppm, respectively [54]; *ca.* 4-fold higher for Glo1 than Glo3. The substrate of Glo1 is the hemithioacetal (HA) adduct of MG with reduced glutathione (GSH) whereas the substrate of Glo3 is MG; from the concentration of GSH in *A. thaliana*, *ca.* 0.4 mM [55], and equilibrium constant for HA formation, 333 M^{-1} [56], the deduced HA/MG concentration ratio is 0.17. Taking these factors into account (3,100 x 4 x 0.17), the ratio of the rate of metabolism of MG *in situ* by Glo1/Glo3 is *ca.* 2,100 or only *ca.* 0.05% MG is metabolized by Glo3. Therefore, Glo3 does not contribute significantly to MG metabolism in plants under physiological conditions and may have a different, as yet, unidentified function. There is also a protein called glyoxalase-4 (Glo4) with no kinetic characteristics reported which appears to have a role in metabolism of high concentrations of exogenous MG (10 mM) [57]. The physiological relevance of putative Glo4 remains to be evaluated – particularly the enzymatic activity of Glo4 with the markedly lower levels of MG levels found in plants under physiological conditions – see Table 3.

The presence of the glyoxalase system in plants has been known for many years [25] where it was initially linked to plant cell growth [58]. Later, it emerged that overexpression of Glo1 and Glo2 in tobacco plants provided increased tolerance to high exogenous MG and high salinity stress [30]. Genomic analysis identified 19 potential Glo1 and four Glo2 proteins in rice and 22 Glo1 and 9 Glo2 proteins in *A. thaliana*. The expression profiles differed in response to abiotic stresses in different tissues and during various stages of vegetative and reproductive development [59]. A more recent study has identified 40% of

Glo1 activity and 10% Glo2 activity in chloroplasts of spinach [60]. In *A. thaliana*, under high CO_2 concentrations where photosynthesis and formation of MG are increased, Glo1 and Glo2 expression and activities were increased. This identifies the function of the glyoxalase system in plants as one fundamental to plant biochemistry: providing a protection against endogenous dicarbonyl stress [60]. Increased expression of Glo1 was involved in the adaptive response of wild type *A. thaliana* and the ascorbate-deficient mutant *vtc2-2* to prolonged exposure to high light intensity [61]. This may be now seen as due to protecting against risk of photosynthesis-linked dicarbonyl stress.

There are AKRs in higher plants [62]**.** These were investigated in *Oryza sativa* (Asian rise) for their role in the anti-glycation defense. AKR isoform-1 of rice (OsAKR1) was induced by abscisic acid and various stress treatments; whereas two other AKR genes were moderately stress-inducible. OsAKR1 is an NAPDH-dependent reductase with catalytic activity towards MG and malondialdehyde; the latter formed in lipid peroxidation. Heterologous expression of OsAKR1 in transgenic tobacco plants produced increased tolerance to oxidative stress generated by methylviologen and improved resistance to high temperature. Transgenic tobacco plants also exhibited higher AKR activity and accumulated less MG in their leaves than the wild type plants, both in the presence and absence of heat stress. These results suggest OsAKR1 may also have a role in cytoprotection against dicarbonyl stress in plants [26].

6. Glycation in plants – considerations for crops and other commercial aspects

B. oleracea is an economically and nutritionally important species of plant that is the product of domestication with limited genetic diversity compared to its wild ancestral relatives. The species exists in several different cultivated forms: cabbage, cauliflower, broccoli, and Brussels sprouts; and also in its wild form distributed along the European

Atlantic seaboard and throughout the Mediterranean area. The reduced genetic base of domesticated *B. oleracea* makes it difficult to find new variants that contribute towards phenotypes capable of resisting stress. Stress resistant varieties are needed to respond to the local and global challenges of food security [63].

Self-incompatibility (rejection of 'self'-pollen) is a reproductive barrier preventing in breeding and thereby promoting plant outcrossing and hybrid vigor [64]. Glo1 is a stigma compatibility factor required for pollination to occur and is targeted by the self-incompatibility system. Decreased Glo1 expression reduced compatibility, and overexpression of Glo1 in self-incompatible *Brassica napus* stigmas resulted in partial breakdown of the self-incompatibility response, suggesting MG-modified proteins may produce a response leading to pollen rejection [65]. Copy number increase of the GLO1 gene has been explored in *Brassica* plants [66]. In future, crop varieties having functional increased copy number of GLO1 may be assessed for improved cross-breeding and improved growth and rigor in maturity and harvest.

Recombinant human proteins may be expressed in plant-based systems. Examples include human serum albumin and protein immunogenic epitopes for vaccine development and production [67, 68]. Recombinant human serum albumins (HSAs) were produced in *O. sativa*. Liquid chromatography-mass spectrometry analysis identified a greater number of hexose-glycated arginine and lysine residues on HSA produced in *O. sativa* than of human plasma origin. There was supplier-to-supplier and lot-to-lot variability in the degree of glycation of HSA expressed in *O. sativa*. Glycation influenced the presence of oligomeric species and tertiary structure of the HSA produced. This may have further implications for the use of HSA expressed in *O. sativa* as a therapeutic product [67]. The relevance of this glycation is not yet clear but if the abnormal glycation impairs function of HSA – such as

ligand binding and esterase activity [69], it may bring into doubt the use of plant produced albumin for clinical applications.

7. Why is glycation potentially damaging to plants?

Glycation in plant proteins is found at relatively low levels: an estimated 26 mol% for FL residues and 4 mol% for MG-H1 residues [1]. Glycation is particularly damaging if it occurs on amino acids in functional domains of proteins and modification produces loss or change of charge of the target amino acid [70]. To assess the probability of glycation sites being in functional domains in the plant proteome, we applied the sequence-based receptor binding domain (RBD) analysis [71] to the proteome of *A. thaliana*. In optimised format, RBD analysis involves a plot of mean hydrophobicity against mean dipole moment of a window of 5 amino acid residues moved sequentially along the sequence of a protein (with a gyration angle between two consecutive residues in the sequence of 100° assumed). This approach had 80% accuracy when validated against a database of known interacting proteins [71]. The outcome of the application of RBD analysis to the proteome of *A. thaliana* is given herein for the first time in Table 4. The prediction of the proportion of amino acid residues of the RBD region or functional domain suggests the amino acid residue targets of glycation, lysine and arginine residues, are enriched in functional domains – 2.1-fold and 3.7-fold, respectively. In contrast, the amino acid targets of oxidative damage are depleted in functional domains: enrichment – cys, 0.8; met, 0.7; tyr, 0.8; and trp, 0.6. The plant proteome thereby is predicted to be relatively resistant to oxidative functional impairment [72, 73]. Glycation of lysine by glucose to form FL residues leads to retention of side chain charge whereas glycation of arginine by MG produces MG-H1 residues and loss of charge. Since arginine residues are often in functional domains for salt bridge electrostatic interaction with other proteins, enzyme substrates and nucleic acids, formation of MG-H1 often produces

protein inactivation and misfolding [6, 70]. The plant proteome is, therefore, susceptible to glycation by MG at functional domains. This is likely an important physiological source of misfolded proteins and substrates for the UPR in plants [74].

8. Role of dicarbonyl stress in the unfolded protein response in plants

Recent studies suggest MG modification of proteins produces misfolding and activation of the UPR [6]. There is an analogous UPR system in plants. It is activated in response to cold, drought, heavy metals and light stress. It preserves proteostasis and protects against inhibition of photosynthesis [74]. The UPR in plants remains to be fully characterized [6, 75]. As in mammalian systems, the inhibitor of enzymatic glycosylation, tunicamycin, has often been used to activate the UPR in the endoplasmic reticulum (ER) or induce ER stress. The physiological activators of unfolded proteins are different: often linked to spontaneous modification such as oxidative damage and MG-derived AGE formation. The latter is particularly damaging to protein structure because it produces loss of charge, modifications are often in folded and highly structured functional domains and because MG modification also inactivates chaperonins which catalyze correct folding of proteins [6]. The link between dicarbonyl stress and the UPR in plants now deserves investigation.

9. Conclusions

Protein glycation is an unavoidable part of plant metabolism and proteotoxicity, contributing to the damaging effects of excess light, environmental and other stresses in plants. Glycation by glucose and MG produces major early-stage glycation adducts and AGEs, respectively. The levels of these glycating agents and related glycation adducts change with developmental stage, senescence, light and dark cycles and also with biotic and abiotic stresses. Proteomics analysis suggests susceptibility of the plant proteome to functional

inactivation by glycation – particularly glycation on arginine residues by MG. Dicarbonyl stress is an abnormal metabolic state, developing in mature plants during normal growth and cultivation. It may be linked to plant self-incompatibility, impaired plant vitality, pre-mature senescence and sensitivity to abiotic stress – including salinity, drought, extreme temperature and prolonged use of ammonium salts. Metabolically, dicarbonyl stress is a driver of increased formation of misfolded proteins and activation of the UPR. Crop breeding for increase functional GLO1 gene copy number in plants may produce varieties resistant to dicarbonyl stress, abiotic stresses and senescence with improved breeding and growth characteristics – including plants of commercial ornamental and crop cultivation values. Metabolic drivers, dicarbonyl metabolites, consequences and a strategy for resolution of dicarbonyl stress in plants are summarized in Figure 2.

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Author contributions

NR and PJT wrote the manuscript, PJT did the dicarbonyl analysis for Table 2 and M.A.-M computed the proteome statistics for Table 4. All authors read and approved the manuscript.

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Conflicts of interest

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Table 1. Early-stage glycation adducts and advanced glycation endproducts.

Glycating agent	Comment
Glyoxal	Formed by the degradation of reducing sugars, glycated proteins, nucleotides and lipid peroxidation [11, 36]. Metabolised by the glyoxalase system [5]. Glyoxal is present in solution mainly as mono- and di-hydrates [76].
Methylglyoxal (MG)	Formed mainly by the trace-level degradation of GA3P and DHAP [17]. Relatively high flux reactive dicarbonyl metabolite. Metabolised by the glyoxalase system [5]. MG is present in solution mainly as mono- and di-hydrates [76]. Precursor of the major AGE, MG-H1.
3-Deoxyglucosone (3-DG)	Formed by the degradation of reducing sugars and glycated proteins. Also formed by enzymatic repair of FL [77]. Metabolised by aldoketo reductases [5]. 3-DG is present in solution as a complex mixture of cyclic hemiacetals and hemiketals [76].
Glycation adduct	**Comment**
N_ε-(1-Deoxy-D-fructos-1-yl)lysine (FL)	Early-stage glycation adduct [1]. Formed from glucose non-enzymatically and reporting on exposure to increased glucose concentration. Repaired intracellularly by fructosamine 3-phosphokinase [78].

Table 1. Early-stage glycation adducts and advanced glycation endproducts (continued).

MG-derived hydroimidazolone (MG-H1)	A major quantitative arginine-derived AGE formed from MG. Influenced by the rate of formation of MG, rate of metabolism of MG by Glo1 of the glyoxalase system and cellular proteolysis. Major AGE in *A. thaliana.* Implicated in protein misfolding and, in excess, activation of the UPR.
N_{ε}-Carboxymethyl-lysine (CML)	A major quantitative lysine-derived AGE. Formed by the oxidative degradation of FL (major), glycation by glyoxal and by ascorbic acid (usually minor). Increased by light stress in *A. thaliana.* CML/FL ratio is a marker of oxidative stress.
Glucosepane	Major quantitative crosslink formed in protein glycation [79]. Formed by the degradation of FL residues with a proximate arginine residue. Content in plant proteins is unknown.
Pentosidine	Low level pentose sugar-derived glycation crosslink and intense fluorophore. Considered to reflect pentosephosphate pathway activity [80].

Table 2. Effect of growth conditions on protein glycation in *Arabidopsis thaliana*.

Growth condition	Effect on protein glycation of glycating	Reference
Daylight to dark growth cycle	Early glycation adduct, FL: 3 mmol/mol lys (daylight entry), increasing to 10 mmol/mol lys (dark).	[1]
Diurnal period, heat, light, and drought	Glycation adducts detected: CML, CMA, FL, G-H1 and MG-H1. Protein targets: a core group of 112 proteins, including chloroplast ATP synthase (β-subunit) and phosphoglycerate kinase. Glycated protein abundances were similar in heat, light, and drought stresses. Glycated proteins with decreased abundance were: light stress – 2 (RPI3 and TPI, decreased); heat stress – 1 (TPI, decreased); diurnal variation – 8 (ASP5, FTSH2 and RAN3, increased AOC2, BAS1, CORI3, OASB, PRK, PRXQ and PURA, decreased); and drought stress 17 (A2, GSA2 and P83484, increased, CAT2, CICDH, CTIMC, CYP18-4, FBP, GGAT1, GLU1, LOX2, P25697, PER34, RBCS-1A, RBCS-3B, TGG2 and TL29, decreased).	[22]
Excess light stress	AGEs increased: G-H1 (0.14 to 0.35 mmol/mol arg) and CML (0.77 to 1.65 mmol/mol lys)	[1]

Table 2. Effect of growth conditions on protein glycation in *Arabidopsis thaliana* (continued).

Growth condition	Effect on protein glycation of glycating	Reference
Osmotic stress	Major glycation adducts detected: CML, CMA and G-H1; 785 glycation sites detected on 724 proteins - 33 and 62 glycation sites were unique for control and osmotically stressed plants, respectively. Abundance changes of AGE-modified proteins in osmotic stress (range - 2-fold decrease to 27-fold increase): 12 proteins involved in lipid metabolism, DNA supercoils and methylation; protein ubiquitination and degradation, energy metabolism, cell organization and development, cell wall formation, regulation of transcription and stress.	[19]
Ammonium NH_4^+ salts	MG-H1 and CEL-modified proteins detected by immunoblotting and immunoassay; 15% increase of CEL in ammonium NH_4^+-grown plants compared with those in nitrate NO_3^--grown control plants	[47]

Table 3. Reactive dicarbonyl glycating agents in *Brassica oleracea* during development.

Days post-sowing	Plant appearance	Dicarbonyl metabolite (nmol/g fresh weight; mean ± SD, n = 6)		
		Glyoxal	MG	3-DG
6		0.38 ± 0.04	2.90 ± 0.81	0.76 ± 0.29
30		0.46 ± 0.12	3.47 ± 1.21	1.80 ± 1.05*
65		0.81 ± 0.32**,OO	4.08 ± 0.27*	0.49 ± 0.23O

B. oleracea leaves were from broccoli cv. GDDH33, a well characterised doubled haploid breeding line derived from cv. Green Duke was sown into F2 compost. The leaves from 6 plants were removed and flash frozen in liquid nitrogen and stored at -20 ^{o}C until analysis. The dicarbonyl contents in the leaves were determined by stable isotopic dilution analysis LC-MS/MS [24]. Briefly, plant leaf (*ca.* 10 mg fresh weight) was homogenised in 5 % trichloroacetic acid with 0.3 % azide to inhibit peroxidase. Internal standards ([$^{13}C_3$]MG, [$^{13}C_2$]glyoxal and ([$^{13}C_6$]3-DG, 2 pmol) were added, mixed and centrifuged (10,000 g, 10 min, 4 ^{o}C). Supernatants were derivatised with 1,2-diaminobenzene and analysed by LC-MS/MS. Significance: * and **, P<0.05 and P<0.01, with respect 6 days and o and oo, P<0.05 and P<0.01, with respect 30 days; *Student's t-test.* Data on MG estimation was published previously [24].

Table 4. Receptor binding domain (RBD) analysis of the proteome of *Arabidopsis thaliana.*

Amino acid	Count		% AA in Proteome	% AA in RBD	Fold Enrichment
	Proteome	RBD			
Ala	463,770	25,941	6.5	3.3	0.5
Arg	380,640	150,922	5.3	19.5	3.7
Asn	317,995	44,745	4.4	5.8	1.3
Asp	384,200	52,528	5.3	6.8	1.3
Cys	130,271	10,915	1.8	1.4	0.8
Gln	250,179	38,180	3.5	4.9	1.4
Glu	474,124	70,661	6.6	9.1	1.4
Gly	473,373	30,225	6.6	3.9	0.6
His	160,243	20,712	2.2	2.7	1.2
Ile	392,264	8,682	5.5	1.1	0.2
Leu	697,276	28,075	9.7	3.6	0.4
Lys	449,328	101,031	6.3	13.0	2.1
Met	164,360	11,802	2.3	1.5	0.7
Phe	314,311	8,387	4.4	1.1	0.2
Pro	341,009	29,637	4.7	3.8	0.8
Ser	636,209	67,405	8.9	8.7	1.0
Thr	369,142	36,395	5.1	4.7	0.9
Trp	90,588	5,539	1.3	0.7	0.6
Tyr	209,664	17,971	2.9	2.3	0.8
Val	487,953	15,139	6.8	2.0	0.3
Total:	7,186,899	774,892	100	100	

Amino acid count and RBD analysis applied to 15,938 reviewed protein sequences of the UniProt Knowledgebase (UniProtKB; www.uniprot.org).

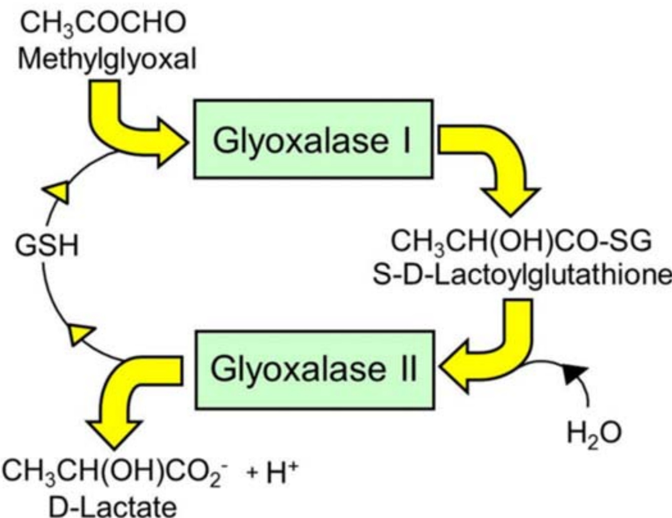

Figure 1. The glyoxalase system. Shown is the metabolism of methylglyoxal to D-lactate.

Glyoxal is metabolised similarly to glycolate.

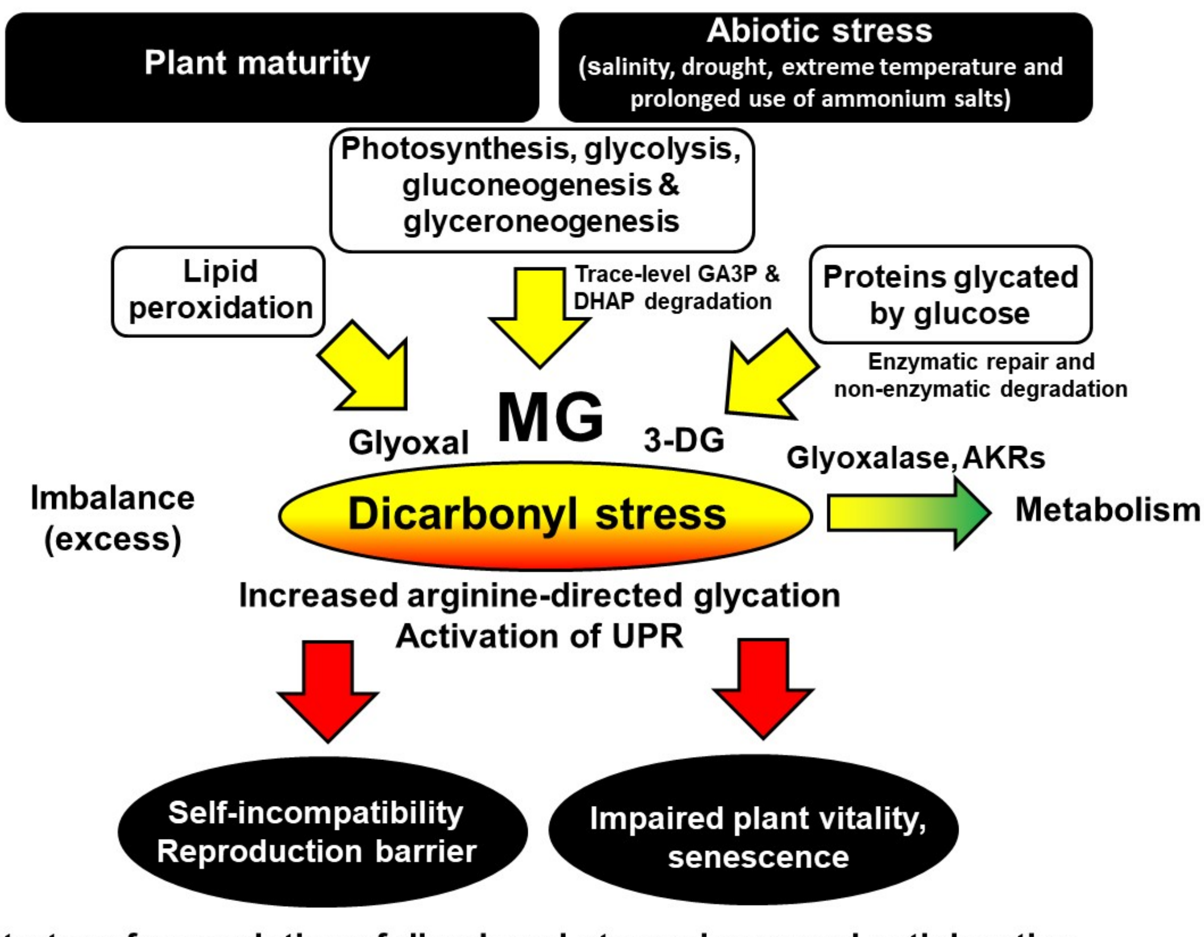

Figure 2. Metabolic drivers, pathophysiological effects and strategy for resolution of dicarbonyl stress in plants.