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In-Silico Analysis & *In-Vivo* Results Concur on Glutathione Depletion in Glyphosate Resistant GMO Soy, Advancing a Systems Biology Framework for Safety Assessment of GMOs

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Abstract

This study advances previous efforts towards development of computational systems biology, *in silico*, methods for biosafety assessment of genetically modified organisms (GMOs). C1 metabolism is a critical molecular system in plants, fungi, and bacteria. In our previous research, critical molecular systems of C1 metabolism were identified and modeled using CytoSolve®, a platform for *in silico* analysis. In addition, multiple exogenous molecular systems affecting C1 metabolism such as oxidative stress, shikimic acid metabolism, glutathione biosynthesis, etc. were identified. Subsequent research expanded the C1 metabolism computational models to integrate oxidative stress, suggesting glutathione (GSH) depletion. Recent integration of data from the EPSPS genetic modification of Soy, also known as Roundup Ready Soy (RRS), with C1 metabolism predicts similar GSH depletion and HCHO accumulation in RRS. The research herein incorporates molecular systems of glutathione biosynthesis and glyphosate catabolism to expand the extant *in silico* models of C1 metabolism. The *in silico* results predict that Organic Soy will have a nearly 250% greater ratio of GSH and GSSG, a measure of glutathione levels, than in RRS that are glyphosate-treated glyphosate-resistant Soy versus the Organic Soy. These predictions also concur with *in vivo* greenhouse results. This concurrence suggests that these *in silico* models of C1 metabolism may provide a viable and validated platform for biosafety assessment of GMOs, and aid in selecting rational criteria for informing *in vitro* and *in vivo* efforts to more accurately decide in the problem formulation

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phase whose parameters need to be assessed so that conclusion on "substantial equivalence" or material difference of a GMO and its non-GMO counterpart can be drawn on a well-grounded basis.

Keywords

Glutathione, Genetic Modification, C1 Metabolism, Biomarker, Safety Assessment, *In-Silico* Analysis, Glyphosate, CytoSolve, Systems Biology, Safety Assessment

1. Introduction

A global public health debate is underway concerning the safety of genetically modified organisms (GMOs). Our laboratory's previous efforts provided, to the best of our knowledge, the first systems biology analysis concluding with the need to establish objective, independent and transparent safety standards for safety assessment of GMOs [1]-[4]. The National Academy of Sciences recent report concurs with our findings to establish such independent, objective and transparent standards for safety assessment of GMOs [5]. In this manuscript, we provide new results that further substantiate our systems biology approach as a viable framework to develop such safety assessment standards for GMOs in a transparent and collaborative manner.

The current "standards" for safety assessment of GMOs are subjective, self-reported and highly opaque [6] [7]. The methodology required by a GMO manufacturer to gain "approval" prior to release of a GMO into the public food supply, at best, is fundamentally unscientific and a process that is not well understood by most citizens and journalists. The word *approval*, in this context, is in double quotes because the methods for "approval", outlined by the US Food and Drug Administration (FDA), are based on self-reporting by the GMO manufacturer [8].

To briefly review this method, the current guidelines are based on the concepts of *substantial equivalence* and/or *material difference* [9]. In this conceptual framework, a GMO manufacturer is given the freedom to select any *criteria* that they deem sufficient to evaluate the "equivalence" or "difference" of a GMO with its non-GMO counterpart [10]. The GMO manufacturer then conducts their own subjective tests, based on using those self-selected criteria, in their own laboratories, without any stipulation that the results from such testing be shared and made accessible to the public [8] [10]. This lack of transparency is significantly different from the detailed and the relatively open level of reporting that the FDA requires from pharmaceutical manufacturers before FDA allowance of drugs for human consumption [9]. Moreover, a drug is further constrained by the fact that only a licensed medical doctor can prescribe the approved drug to a patient. In the case of GMOs, anyone can buy and consume the GMO following "approval".

At present, the GMO manufacturer is only required to inform the FDA that they have completed their internal testing of substantial equivalence or material difference. The FDA, after being informed that such testing has been completed by the GMO manufacturer, then executes a non-mandatory "safety consultation" and issues a letter to the GMO manufacturer to memorialize that the GMO manufacturer has made statements to the FDA as to having conducted such measurements. A simple review of a sample safety consultation letter issued by the FDA makes the perfunctory nature of this "approval" process amply clear [11]. In this process, the FDA does not require any independent or objective testing to validate the GMO manufacturer's self-reported results. In short, there are no objective standards for safety assessment of GMOs. In the current paradigm, there is no mandatory third-party independent testing required to verify the self-reported results of the GMO manufacturer.

Only recently has the broader scientific community recognized the egregious flaws of the current "approval" process. The recent National Academy of Sciences (NAS) report exemplifies this growing recognition. For example, concerning the need for third-party independent testing of GMOs, the NAS report is unequivocal: "Finally, an important effect of a regulatory system is to enable markets by creating a credible and independent process to verify that products are safe. As noted in Chapter 2, publics in many countries, including the United States, are wary about the safety of GE crops and foods. There should be concern about the effect on public opinion if GE crops and foods are brought to market without government review for safety. Without the assurance that there has been some third-party review for safety, consumers' perceptions about the safety of GE food and crops might erode completely. Although consumer confidence should not be the only rationale for a product-

approval system, it is important to recognize that it is an important social and economic factor" (pg. 341 in [5]).

The report concludes that, "Not having government regulation of GE crops would be problematic for safety, trade, and other reasons and would erode public trust" (pg. 341 in [5]).

The NAS report further highlights the need for transparency in the process of GMO safety: "Accuracy and trust are critical for technology governance. The committee renews the advice from prior National Research Council reports to regulatory agencies to expand efforts to include the public in their deliberations and to make their decisions and the information on which they base their decisions as transparent as possible, recognizing the constraints of various laws that protect confidential business information and other sensitive data. Similarly, the committee emphasizes that governance authorities should actively seek public input on decisions, including decisions regarding how to approach emerging genetic-engineering technologies (such as genome editing and synthetic biology) and their regulation" (pg. 341 in [5]).

Relative to ensuring accuracy in reporting, the NAS report also recommends that, "In cases in which early published studies produced equivocal results regarding health effects of a GE crop, follow-up experimentation using trusted research protocols, personnel, and publication outlets should be used to decrease uncertainty and increase the legitimacy of regulatory decisions. Public funding in the United States should be provided for independent follow up studies when equivocal results are found in reasonably designed initial or preliminary experimental tests" (pg. 130 in [5]).

Our laboratory's efforts concerning GMO safety assessment has been oriented towards the development of a foundational systems biology framework that is non-reductionist and systematically integrates multiple independent research findings, across multiple institutions, in a transparent and collaborative manner, to understand the nature and extent of perturbations in molecular mechanisms and pathways following genetic engineering. The current notion of safety is based on the assumption that GMOs are not materially different from their non-GMO counterpart using arbitrary and self-selected criteria. Our systems biology approach provides a quantitative analytical framework to determine objective criteria for assessing the substantial equivalence or material difference of a GMO and its non-GMO counterpart.

The development of such a systems biology framework is in alignment with other recommendations within the NAS report [5] that recognize that, "…the [current] process-based approach has become less and less technically defensible as the old approaches to genetic engineering become less novel and the emerging processes fail to fit old categories of genetic engineering. Moreover, because the emerging technologies have the potential to make both incremental changes that lack substantial risk and major changes that could be problematic, the committee recommends that a tiered approach to regulation should be developed that uses trait novelty, potential hazard, and exposure as criteria. -Omics technologies will be critical for such an approach."

1.1. CytoSolve: Advanced -Omics Technology Framework for GMO Safety Assessment

Towards adopting such advanced -omics technologies, our laboratory has employed CytoSolve as the core operating system for building a framework for GMO safety assessment. In earlier work, spanning four other publications [1]-[4], CytoSolve was used to develop a quantitative molecular systems understanding of C1 metabolism—a critical system of molecular pathways inherent to all plants, fungi and bacteria. The CytoSolve technology (discussed in detail within the Methods section herein) provides a proven and systematic approach to integrate the molecular systems identified in disparate and diverse wet laboratory (*in vitro* and *in vivo*) experiments. In that previous work, molecular pathways identified in 6529 wet laboratory experiments, spanning 184 scientific institutions, across 23 countries were quantitatively integrated using CytoSolve to conclude that the GMO Soy was not substantially equivalent, but materially different, from its non-GMO Soy counterpart, particularly relative to the levels of glutathione (GSH) and formaldehyde (HCHO) within C1 metabolism system [1]-[4].

The CytoSolve technology and approach is a well-established framework for quantitative and predictive modeling and integration of molecular pathway systems. CytoSolve has been published and cited in eminent journals such as IEEE, Nature Biotechnology, Nature Neuroscience, CELL's Biophysical Journal, and others [12]-[16]. CytoSolve has been employed in many other scientific problems beyond GMO safety, following its early development starting in 2003 at the Massachusetts Institute of Technology (M.I.T). In cardiovascular research, for example, CytoSolve has been used to accurately model nitric oxide (NO) production in endothelial cells subjected to shear stress [15]. In neurovascular studies, the CytoSolve process elicited and derived a novel engineering systems architecture demonstrating the commonality of multiple neurovascular diseases as communica-

tion dysfunctions in common molecular signaling sub-systems and components [14]. In oncology, CytoSolve's unique capability for *in silico* modeling to derive multi-combination therapeutics has been independently recognized by leading cancer researchers [12]. The FDA has also issued allowance for CytoSolve to proceed to clinical trials for a multi-combination therapeutic derived from CytoSolve's *in silico* modeling of pancreatic cancer [17].

In summary, CytoSolve fundamentally provides a 21st century -omics technology for quantitative integration of disparate research findings in a transparent and collaborative manner to gain a holistic and systems-based understanding of complex molecular phenomenon. This capability of CytoSolve's, in particular, provides an important and critical tool to address the various gaps for GMO safety identified in the NAS report.

1.2. The Need for Objective Criteria Selection in Determining Substantial Equivalence and Material Difference of a GMO with Its Non-GMO Counterpart

One of the critical gaps for assessing safety of GMOs is to determine what criteria are appropriate to assess the substantial equivalence or material difference of a GMO and its non-GMO counterpart. As aforementioned, the current process of identifying such criteria is self-selected by the GMO manufacturer. Why and how such criteria are selected is neither based on objective standards nor on any cogent or detailed understanding and analysis of molecular mechanisms. CytoSolve provides a novel framework to model complex molecular mechanisms to systematically identify such criteria.

Our laboratory has been leading the effort in using CytoSolve to determine such objective criteria using a *systems biology* approach. Systems biology aims to understand the complexity of the whole organism, as a system, rather than just studying its parts in a reductionist manner. This systems-based approach can enable the determination of such criteria, as it recognizes that genetic modification, small or large, may affect emergent properties of the whole system [18]. Previously, our laboratory's *in silico* analysis has shown that genetic insertion of the CP4 EPSPS gene in *Glycine max* L. (Soy), commercially known as Roundup Ready Soy (RRS), perturbed five molecules within the C1 metabolism molecular system, resulting in substantial difference in the levels of over twenty (20) molecular species in GMO Soy compared to the non-GMO Soy, one of them being glutathione [4]. The results from that previous research predicted significant accumulation of formaldehyde and concomitant depletion of glutathione in the GMO, suggesting how a "small" and single GM creates "large" and systemic perturbations to molecular systems equilibrium. Our previous work recommended that regulatory agencies, currently reviewing rules for GMO safety, may wish to adopt a systems biology approach using a combination of *in silico*, computational methods and subsequent targeted experimental *in vitro* and *in vivo* designs, to develop a systems understanding of "equivalence" using biomarkers, or *criteria*, such as formaldehyde and glutathione, which predict metabolic disruptions, towards modernizing the safety assessment of GMOs.

In this current effort, we are focused on exploring, the merits of employing glutathione, in particular, as a viable criteria for safety assessment of GMOs. Glutathione is one of nature's master anti-oxidants that occurs in nearly all life forms and is important for maintaining the redox homeostasis [19]. Towards this end, in this research, we expand our previous *in silico* model to incorporate glutathione biosynthesis as well as glyphosate metabolism to more accurately model the perturbations caused in RRS, the GMO Soy. The *in silico* model is then used to assess the differences in level of glutathione in RRS versus its non-GMO counterpart, Organic Soy. In this study, our purpose, more importantly, is to validate the disruption predicted by our *in silico* models by identifying independent third-party wet laboratory, *in vitro* or *in vivo* experimental studies that have also observed such disturbance in glutathione in RRS versus Organic Soy.

2. Background

Oxidative stress at the cellular level is capable of causing profound alterations of various biological structures, including cellular membranes, lipids, and nucleic acids and numerous biochemical processes. It is a process that can lead to numerous types of malignancies. Reduced glutathione (GSH) is considered to be one of the most important scavengers of reactive oxygen species (ROS), and its ratio compared to oxidized glutathione (GSSG) may be used as a marker of cellular oxidative stress [20].

The current version of the integrative *in silico* model of C1 metabolism in GMO Soy, specifically in RRS, predicts complete depletion of glutathione and accumulation of formaldehyde as a result of oxidative stress [4]. However, *in vivo* wet lab experiments have demonstrated that glutathione levels are not completely depleted but

fluctuate to new steady state levels, be it during genetic modification such as in RRS or during drought conditions [5]-[8]. Plants undergoing oxidative stress produce oxidized glutathione (GSSG) from the reduced form of glutathione (GSH). Hence GSH/GSSG ratio is an accepted biomarker of oxidative stress in plants [21].

An earlier systematic bioinformatics literature review identified critical molecular pathway systems involved in C1 metabolism. The C1 metabolic process within cells provides one-carbon units for proteins, nucleic acids, methylated compounds, and other biomolecules. C1 metabolism is found in plants, bacteria, yeast, and mammals. In this initial work [1] three critical molecular systems involved in C1 metabolism were identified as shown in **Figure 1**. Such identification provided the basis for the development of an initial *in silico* computational model that integrates these three systems of C1 metabolism [2].

That initial work resulted in a computational model, which, to the best of our knowledge, is the first computational model to predict the interrelationships of the various molecular species across the three biochemical processes of C1 metabolism: methionine biosynthesis, activated methyl cycle and formaldehyde detoxification [2]. The individual models of C1 metabolism predict temporal behavior of key molecules in C1 metabolism such as formaldehyde (HCHO), formate, sarcosine and glutathione (GSH). The integrated model of C1 metabolism predicts that glutathione levels are minimally affected and maintain a steady state and formaldehyde is evanescently produced and detoxified rapidly [2].

In subsequent research, while the initial model of C1 metabolism provided important insights, other exogenous molecular systems affecting C1 metabolism were also identified, from using and combining the results from other scientific literature [2]. C1 metabolism from a systems perspective is not an isolated system. In that research, a systems architecture map, as shown in **Figure 2**, was developed to provide a blueprint for modular integration of other systems to evolve and advance the C1 metabolism model. Such exogenous systems include: tetrahydrofolate (THF) biosynthesis, oxidative stress metabolism, catalase activity, shikimic acid metabolism, adenosine metabolism, glyphosate metabolism, formate biosynthesis, and serine biosynthesis, for example [2].

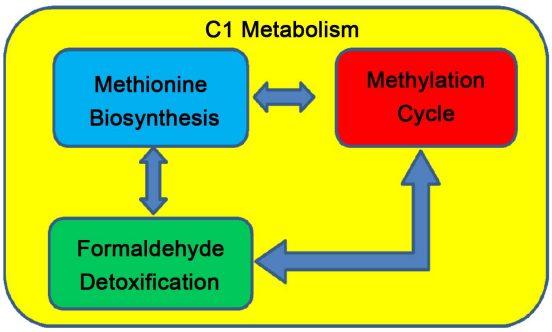


Figure 1. Key molecular systems of C1 metabolism [1].

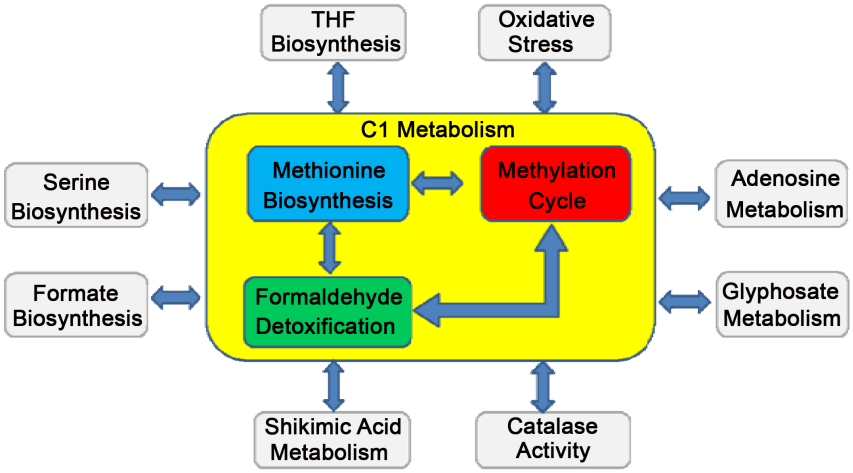


Figure 2. Key molecular systems of C1 metabolism and its interactions with other critical plant molecular systems [2].

Moreover, the research identified the critical sub-systems of the oxidative stress system, and integrated oxidative stress models with C1 metabolism. **Figure 3** shows the computational systems architecture of oxidative stress and C1 metabolism [3]. Interactions of oxidative stress system with C1 metabolism were computationally analyzed to understand the effect of oxidative stress on C1 metabolism.

This molecular systems integration provided two important results:

1) Demonstration of the modular expandability of the then-existing *in silico* model of C1 metabolism to support systems integration of other related molecular pathway systems with ease and scalability; and,

2) Derivation of new insights on the effects of oxidative stress on C1 metabolism relative to formaldehyde (HCHO), a toxic molecule, and glutathione (GSH), an important indicator of oxidative homeostasis in living systems.

This computational molecular systems integration of oxidative stress with the fully integrative model of C1 metabolism predicted that oxidative stress depletes glutathione leading to a concomitant accumulation of formaldehyde.

The possibility of using this integrative model to understand the effects of genetic engineering was subsequently explored to develop a *in silico* platform for informing *in vitro* and *in vivo* efforts for biosafety assessment of GMOs [4]. In that exploration, the effects of perturbations due to genetic insertion of CP4 EPSPS in *Glycine max L.* (Soy) were integrated with an integrative model of C1 metabolism and oxidative stress (two molecular systems critical to plant function) and were compared with control [4]. The computational systems architecture of this integration is shown in **Figure 4**.

The results from this exploration predict depletion of glutathione in the RRS and a concomitant accumulation of formaldehyde.

3. Methods

The research herein aims to advance the current *in silico* model of C1 metabolism by using CytoSolve to modularly integrate other known molecular pathway systems to deepen our mechanistic understanding of the effects

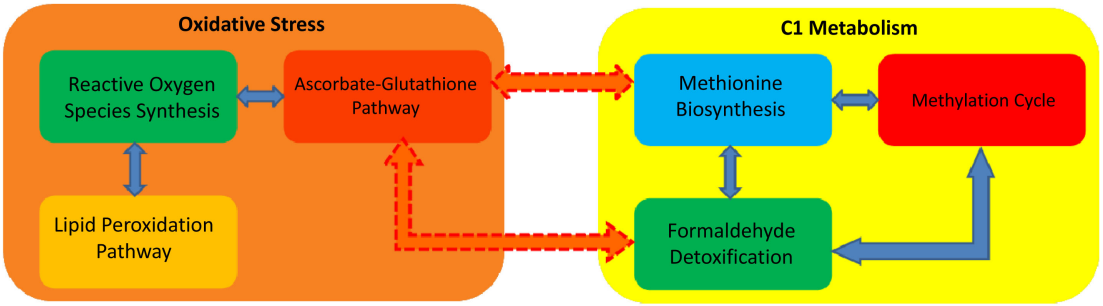


Figure 3. Systems architecture of C1 metabolism pathway with oxidative stress [3].

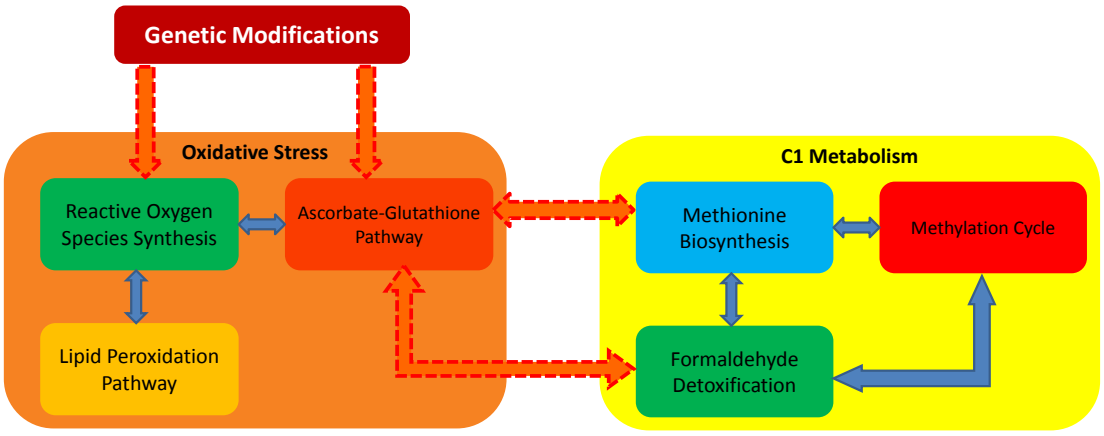


Figure 4. Systems architecture of GM of soy on oxidative stress system and C1 metabolism [4].

of genetic modification, particularly in RRS. In this effort, two specific molecular systems are integrated:

1) Glutathione biosynthesis; and,

2) Glyphosate catabolism

Such integration will expand the previous *in silico* model to include a source of glutathione to more accurately reflect biological activity. In addition, glyphosate catabolism will be integrated since RRS is grown in an environment of glyphosate. The mechanisms of glutathione biosynthesis and glyphosate catabolism, therefore, will be integrated and simulated to predict the GSH/GSSG ratio. This ratio will be compared with existing *in vivo* experimental data to assess the validity of the expanded model for continued advancement towards developing an *in silico* platform for biosafety assessment of GMOs using CytoSolve.

CytoSolve aggregates existing peer-reviewed scientific literature and mines this literature to extract molecular pathways of biological processes [9] [10]. The platform abstracts complex cellular functions as a plurality of such molecular pathways, each of which can be treated as individual models, as illustrated in **Figure 5**.

The CytoSolve platform computationally integrates the individual molecular pathway models, each of which may span multiple spatial and temporal scales, across compartments, cell types and biological domains using the computational framework that enables coupling individual molecular pathway models dynamically without the need to create a monolithic model.

This approach allows for an inherent scalability to build models of complex biological phenomena. The CytoSolve framework provides a mechanism not only for making predictions of complex molecular interactions and behavior but also for informing intelligent *in vivo* and in *vitro* experimental designs to verify such predictions.

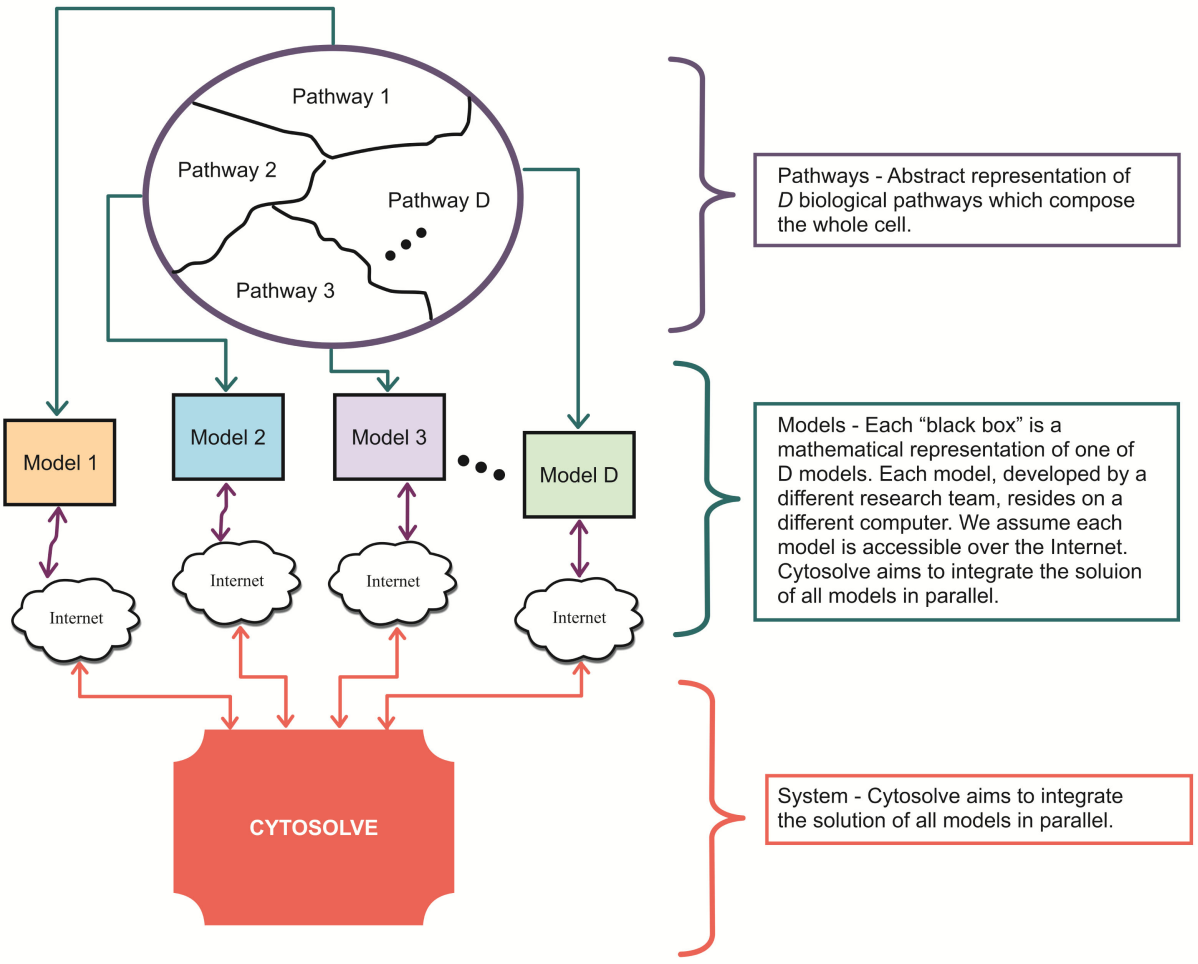


Figure 5. CytoSolve provides a framework for integrating systems of systems of molecular pathway models [22].

3.1. Expansion of Current *In-Silico* Model of C1 Metabolism and Its Validation and Comparison with *In-Vivo* Research

There are six (6) steps undertaken to expand the current *in silico* C1 metabolism model to include glutathione biosynthesis and glyphosate catabolism, and to validate its results with extant *in vivo* data.

First, a systematic bioinformatics literature review is conducted to discover any molecular mechanisms affected by GMSoy. Literature collection from an informatics standpoint is executed to ensure high recall to acquire the initial set. Based on the research questions of "What are the molecular mechanisms involved in glutathione biosynthesis?" and "What effect does glyphosate have on C1 metabolism via oxidative stress in genetically modified plants?" 17 search criteria were developed and are listed in Supplementary Materials' Appendix A. Online databases including PubMed and Google Scholar were searched using the search criteria. An initial set was produced as a result of 17 parallel independent searches. The initial set was searched by constraining the search criteria within the Titles or Abstracts to the following keywords: genetic modification, oxidative stress, glutathione, biosynthesis, glyphosate and C1 metabolism, in plants to acquire the relevant set. The above keywords were used individually, in combination of two or more, and all together while performing the literature search.

The papers from relevant set are reviewed by domain experts to determine the *study set* paper, from the relevant set, containing molecular pathway information such as:

1) Cellular compartments containing species and reactions,

2) Kinetics parameters oxidative stress pathways,

3) Fold-changes in relevant enzymes and key molecular species concentrations.

In this detection process, priority is given to those articles which are the most recent and which contained information and/or studies on glutathione biosynthesis, glyphosate catabolism and Soy. The final step of this literature review is to discover the dynamics of molecular interactions induced by glutathione biosynthesis and glyphosate catabolism in Soy.

Second, any dynamics of molecular interactions, induced bygenetic modification and glyphosate, identified from the literature review, are incorporated to expand the *systems architecture* for glutathione biosynthesis, glyphosate catabolism and C1 metabolism, developed in earlier work [4].

Third, the updated systems architecture is used as the blueprint to create an integrative model of how glutathione biosynthesis and glyphosate catabolism interactwithC1 metabolism in the genetic modification of Soy.

Fourth, the resultant model is used to execute simulations to observe GSH/GSSG ratios for RRS and Organic Soy. All simulations are executed for a simulation time period of 200,000 seconds (~2 days) to make sure the key biomolecular species achieve steady state.

Fifth, literature review is executed to find any research papers that have conducted any *in vivo* experiments to study the effect of glyphosate on GSH/GSSG ratios relative RRS versus Organic Soy Such *in vivo* data can be valuable to compare the results of the expanded *in silico* model of C1 metabolism. Literature collection from an informatics standpoint is executed to ensure high recall to acquire the initial set. Based on the research question of "How do genetic modifications in Soy affect the glutathione levels?" eight (8) search criteria were developed and are listed in Supplementary Materials' **Appendix A**. Online databases including PubMed and Google Scholar were searched using the search criteria to obtain an initial set of literature. The initial set was searched by constraining the search criteria within the Titles or Abstracts to the following keywords: genetic modification, oxidative stress, greenhouse study, *in vivo* experiments, glutathione, GSH/GSSG ratio, glyphosate, Soy and C1 metabolism, to acquire the relevant set.

Sixth, *in silico* results for GSH/GSSG ratios are compared with those from *in vivo* results obtained from the literature.

4. Results

The outcomes of this research are two-fold:

1) An integrative computational model which allows for the study of molecular mechanistic differences between RRS that is glyphosate-resistant and glyphosate-sensitive Organic Soy; and,

2) Simulation results using this integrative model suggest that Organic Soy will have a nearly 250% greater GSH/GSSG ratio than RRS. Specifically, six sets of results emerge from this study.

4.1. Results from Systematic Bioinformatics Literature Review for Glutathione Biosynthesis and Glyphosate Catabolism Effect on C1 Metabolism

A systematic bioinformatics literature review was conducted for the identification of molecular pathways involved in GMO crops, similar to the method used to identify the key molecular pathways of C1 metabolism [1] [2]. Based on the framing of the research question and the application of the search criteria, in Appendix A, through a parallel strategy, the literature collection of an initial set of 242 papers is identified from online databases such as PubMed and Google Scholar. The final results of the systematic review are summarized in **Figure 6**, which identified the critical mechanisms in which glyphosate is metabolized and how the metabolites affect C1 metabolism.

4.2. Identification of Mechanisms of Glyphosate Catabolism and Glutathione Synthesis and Its Effect on Molecular Interactions in Genetic Modification of Soy

Among 79 papers of the reviewed set from the systematic bioinformatics literature review yielded important insights, in particular, on the molecular interactions of glyphosate with C1 metabolism in RRS.

A number of studies on monocotyledonous and dicotyledonous plants have revealed that a metabolism capable of degrading glyphosate to AMPA is either missing or so weak that it can hardly account for plant resistance or tolerance. However, velvet bean (*Mucuna puriens*) exhibits innate resistance to glyphosate due to the presence of pathways degrading glyphosate to AMPA, glyoxylate, sarcosine and formaldehyde [23]. Glyphosate catabolism (degradation), as shown in **Figure 7(a)**, occurs through two pathways. One pathway leads to the intermediate formation of sarcosine and glycine, and the other leads to the formation of AMPA.

In the sarcosine pathway, the initial step is the cleaving of the C-P bond by C-P lyase, producing phosphate and sarcosine. Sarcosine is further degraded to glycine and formaldehyde by sarcosine oxidase.

In the AMPA pathway, the first step is the cleavage of the C-N bond by the enzyme glyphosate oxidoreductase, producing AMPA and glyoxylate. Glycine oxidase from *B. subtilis* can also metabolize glyphosate into AMPA and glyoxylate [24]. AMPA is cleaved to produce inorganic phosphate and methylamine. Although it has been stated that methylamine is ultimately mineralized to CO_2 and NH_3, [25], the presence of methylamine dehydrogenase in soil bacteria can convert methylamine to formaldehyde [26]. The specific kinetics, relative to the dynamics of these five biomolecules and their molecular interactions, are derived from the literature and provided in the *Supplementary Materials* in **Table B1** along with the references. The glyphosate metabolism is shown in **Figure 7(a)**.

Glutathione is a low molecular weight thiol with functions in detoxification, anti-oxidant biochemistry and redox homeostasis. It is synthesized in plants in two ATP-dependent steps, from its constituent amino acids. Synthesis of GSH takes place in two steps-glutamate and cysteine are combined by γ-glutamylcysteinylsynthetase (encoded by GSH1 gene) to form γ-glutamylcysteine. This is the rate-limiting step of GSH synthesis. Glycine is combined with γ-glutamylcysteine to produce GSH, catalyzed by glutathione synthetase (encoded by GSH2). The first step of glutathione biosynthesis, as shown in **Figure 7(b)**, takes place in the plastids while the

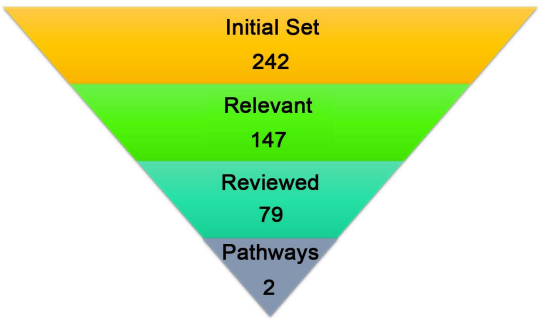
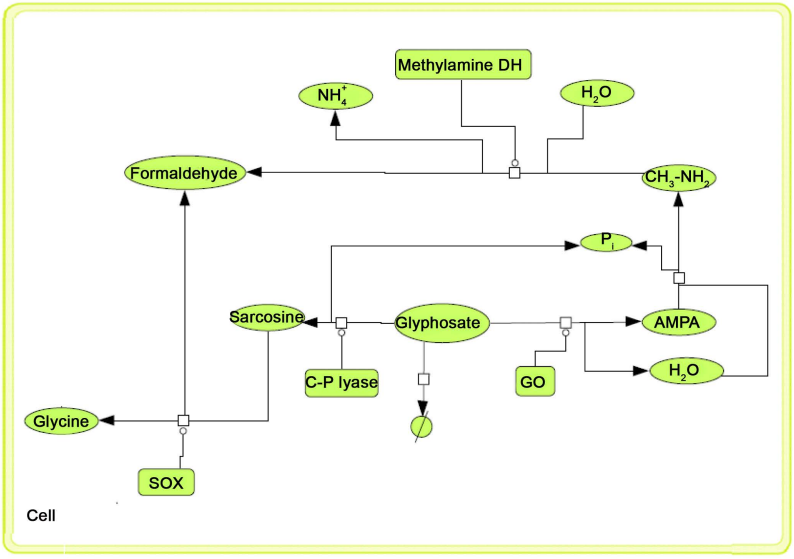
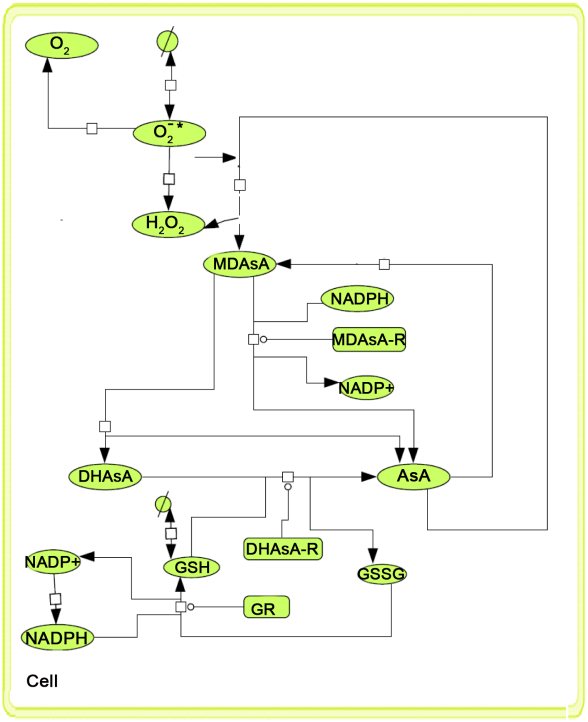


Figure 6. Systematic review results. There are 242 scientific papers (initial set), which met the search criteria. Of those, 147 papers (relevant set) appeared to be relevant based on the title and abstract. Upon further review, 79 papers (study set), were chosen as the study set upon which this work is based. With this study set, two critical mechanisms (final set) were identified. The two mechanisms identified were: 1) glutathione biosynthesis that relates to the formation and degradation of glutathione in C1 metabolism and 2) glyphosate catabolism that related to the breakdown of glyphosate in C1 metabolism.



(a)



(b)

Figure 7. (a) Glyphosate catabolism in plants. The rectangles represent enzymes and elliptical shapes represent other biomolecules. The figure is modified from Kothandaram *et al.* (2015) to incorporate glyphosate catabolism [2]. *SOX—sarcosine oxidase*; *P_i—inorganic phosphate*; *O_2—oxygen*; *C-P lyase-carbon-phosphate lyase*; *NH_4^+—ammonium ion*; *GO—glycine oxidase*; *Methylamine DH—methylamine dehydrogenase*; *H_2O_2—hydrogen peroxide; AMPA—aminomethylphosphonic acid*; *CH_3-NH_2—methyl amine*; (b) Glutathione biosynthesis in plants. The rectangles represent enzymes and elliptical shapes represent other biomolecules. The figure is modified from Mohan *et al.* (2015) to incorporate glyphosate catabolism [3]. *NADP+—nicotinamide adenine dinucleotide phosphate*; *NADPH—reduced nicotinamide adenine dinucleotide phosphate*; *GSH—glutathione*; *GR—glutathione reductase*; *DHAsA—dehydroascorbic acid*; *DHAsAR—dehydroascorbic acid reductase*; *AsA—ascorbic acid*; *O_2^{-*}—superoxide anion*; *H_2O_2—hydrogen peroxide*; *MDAsA—monodehydroascorbate*; *MDsAR—monodehydroascor-bate reductase.*

second step is predominantly cytosolic. Although other factors such as glycine and ATP affect the synthesis of glutathione, the major factors are γ-glutamylcysteinylsynthetase activity and cysteine availability. GSH1 and GSH2 genes are responsive to light, drought and certain pathogens. In addition, γ-glutamylcysteinylsynthetase is regulated by feedback inhibition by GSH. Degradation of glutathione or its conjugates could be catalyzed by carboxypeptidase or phytochelatin synthase. γ-Glutamyltranspeptidases catalyze the hydrolysis or transpeptidation of GSH at the plasma membrane. The products are further processed by γ-glutamylcyclotransferase to produce free glutamate [19].

Glutathione is not produced at equivalent rates by all tissues. Trichomes which are specialized structures on the epidermis show higher expression of enzymes involved in GSH biosynthesis than the surrounding epidermal cells, for instance (Noctor *et al.*, 2002). The cytosol and chloroplasts account for about 50% and 30% of total glutathione in Arabidopsis, although various studies indicate high concentrations of glutathione in mitochondria. Peroxisomes contain glutathione and glutathione reductase, but lack the enzymes of glutathione synthesis. Various transporters are involved in translocation of glutathione between subcellular compartments. Transport across the plasmalemma is achieved by oligopeptide transporter (OPT) family. Inner chloroplast envelope transporters (CLT1, CLT2, CLT3) export γ-glutamylcysteine from the chloroplast to the cytosol for conversion to GSH, which is then transported into the chloroplast. ATP-binding cassette transporters may clear GSSG from the cytosol [19]. Inter-compartmental variations in glutathione concentrations may be crucial in signaling [27].

Turnover kinetics of GSH depends not only on the synthesis and degradation rates, but also on the rates of translocation across various subcellular organelles. In our integrated models of C1 metabolism, oxidative stress and glyphosate detoxification, we have optimized the synthesis and degradation rates of GSH considering such additional factors, in order to validate the overall impact of GSH. The glutathione metabolism is shown in **Figure 7(b)**.

4.3. Systems Architecture of Glyphosate Effect and Glutathione Synthesis on Oxidative Stress and C1 Metabolismin GM Soy

The literature review of the effect of glyphosate on genetic modification in soy and its effects on molecular pathways in previous section provides valuable information on the interface of glyphosate catabolism and glutathione biosynthesis with key molecular species in C1 metabolism. In **Figure 8**, an integrative molecular systems architecture is presented by coupling the dynamics of the molecular interaction in the heretofore known literature, accessible and aggregated by the authors, with the systems architecture of genetic modification, oxidative stress and C1 metabolism derived in earlier work [3].

As shown in **Figure 8**, glyphosate interacts with C1 metabolism by interfacing through the methionine biosynthesis pathway, which interfaces with both methionine biosynthesis and formaldehyde detoxification path-

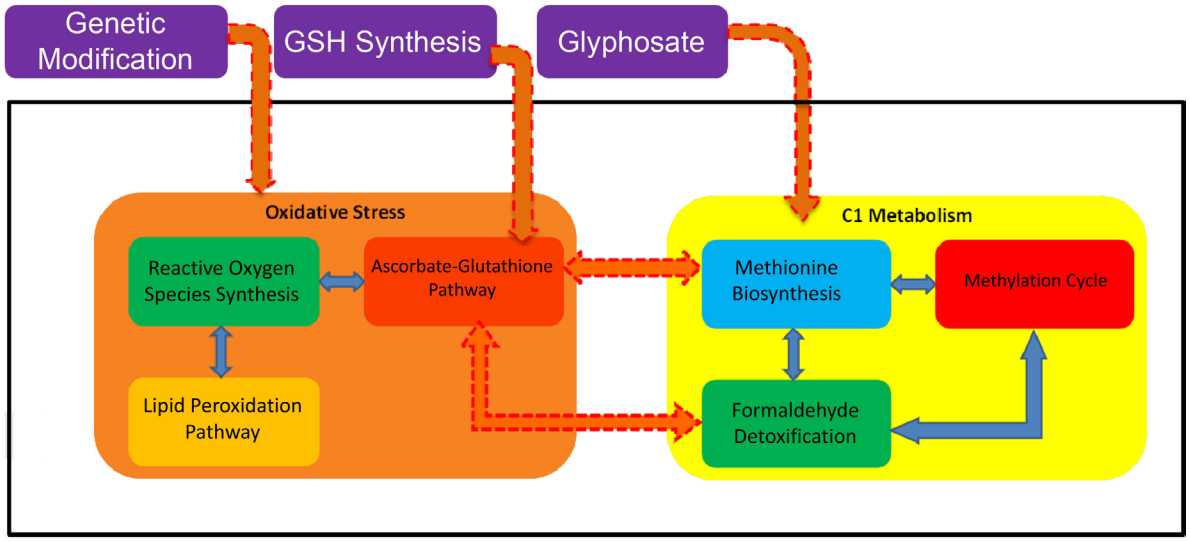


Figure 8. Systems architecture of C1 metabolism interactions with glyphosate.

ways of C1 metabolism. Glutathione synthesis interacts with the ascorbate-glutathione pathway within the oxidative stress system. These interfaces will be relevant in developing and testing the *in silico* modeling of the effects of glyphosate catabolism and glutathione synthesis on C1 metabolism

4.4. Modeling Results of the Ratio of GSH/GSSG Ratio in Organic Soy and RRS

Glutathione is present in reduced form (GSH) in the plants. The oxidative stress caused by genetic modification oxidizes the glutathione levels in C1 metabolism to generate oxidized glutathione (GSSG). GSH/GSSG ratios are predicted for two cases: 1) Organic Soy, and 2) RRS. The simulation results for both cases are shown below.

4.4.1. Simulation Results from *In-Silico* Modeling for Organic Soy

The results obtained from this integrative model reveal the temporal dynamics of GSH/GSSG ratio for Organic Soy as shown in **Figure 9**.

The ratio is higher in the beginning period of simulation as there is more GSH that GSSG and then it reduced and achieved a steady state value of 9.7 for the Organic Soy after 40,000 s. The simulations are executed for a total simulation time period of 200,000 s (~2 days).

4.4.2. Simulation Results from *In-Silico* Modeling for RRS

The results obtained from this integrative model reveal the temporal dynamics of GSH/GSSG ratio for RRS, in presence of glyphosate, as shown in **Figure 10**.

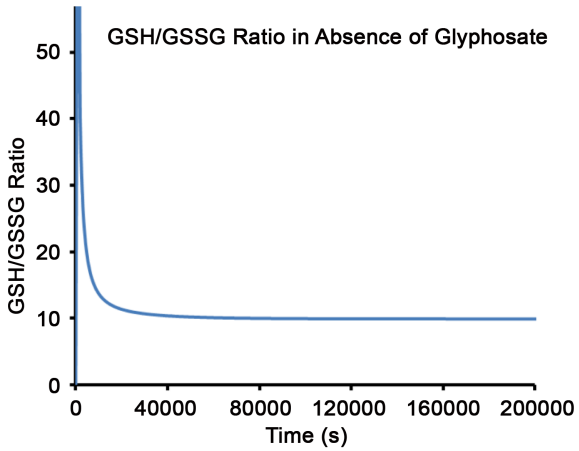


Figure 9. Simulation results of GSH/GSSG ratio in organic soy without glyphosate present.

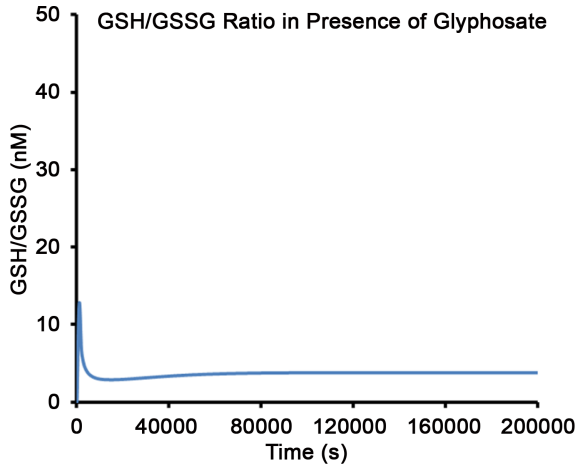


Figure 10. Simulation results of GSH/GSSG ratio in RRS.

The ratio is higher in the beginning period of simulation as there is more GSH than GSSG and then it reduced and achieved a steady state value of 3.9 for the glyphosate-resistant RRS after 40,000 s. The simulations are executed for a total simulation time period of 200,000 s (~2 days).

4.5. *In Vivo* Experimental Findings of GSH/GSSG Ratio in RRS versus Organic Soy

The literature search for in vivo experimental studies measuring the GSH/GSSG ratio in RRS yielded several reports that analyzed the effect of genetic modification on GSH/GSSG ratios [19] [20] [28] [29]. Vivancos *et al.* specifically provides *in vivo* experimental data on GSH/GSSG ratios in RRS and Organic Soy in controlled-environment chambers until the fourth leaf stage, at day/night temperatures of 24˚C/19˚C with a 12-h-day/12-h-night cycle and were watered twice a day. For the glyphosate treatments, 20 mL of Clinic Ace (41.5% glyphosate plus 8.1% Tallow alkylamine ethoxylate; Nufarm) was used in a total 900 mL of water solution [28].

Vivancos *et al.* performed studies of photosynthesis, proteome profile changes, amino acid profiles and redox profiles in these glyphosate sensitive and resistant Soys, both treated with glyphosate. In the sensitive phenotype, photosynthesis was inhibited, a nitrogen-rich amino acid profile was observed, and an increase in defense-associated proteins was triggered. However, there was no evidence of oxidation of cellular redox pools in the sensitive plants. Resistant plants displayed relatively less change in amino acid metabolism and photosynthesis, although there was increased oxidation of redox pools accompanied by a decrease in the ratios of GSH/ GSSG. The authors infer that the accumulation of higher levels of glyphosate in resistant plants enhanced cellular oxidation, possibly by stimulation of photorespiration [28].

In sensitive plants, the observed increases in the total leaf GSH pool suggest that up-regulation of this synthesis pathway is required to allow GSH-dependent glyphosate detoxification pathways. The authors suggest that it is possible that the insensitivity of EPSPS to glyphosate in the resistant genotype decreases the ability of the plant to trigger glyphosate detoxification pathways. Thus, the herbicide accumulates to a level that triggers oxidative stress [28].

Glyphosate leads to oxidative stress in plants. This is due to a secondary effect of the blocked shikimate pathway. Oxidative stress in *P. sativum*, wheat (*Triticum aestivum*) and maize was observed in plants exposed to glyphosate [21]. In maize, glyphosate application increased the levels of lipid peroxidation, glutathione, free proline content and ion flux [21]. In rice leaves, glyphosate application generates hydrogen peroxide, which results in peroxidation and destruction of lipids. In pea plant, glyphosate application on both leaves and roots resulted in the activation of GSH reductase and enhancement of the GST activities [21].

Table 1 shows the comparative analysis of effect of glyphosate on the glutathione and ascorbate levels in the leaves of glyphosate-sensitive and glyphosate-resistant Soy plants.

4.6. Comparative Analysis: *In Silico* versus *In Vivo*

A study of redox profiles in glyphosate sensitive and resistant Soy both treated with glyphosate was conducted to show the effects of glyphosate on the redox pools in the plants [28]. These laboratory measurements, taken 5 days after glyphosate application, showed that in the absence of glyphosate, the glutathione pools were highly (over 90%) reduced. The total glutathione pool of the resistant leaves was oxidized as a result of the glyphosate treatment, and there was a marked decrease in the GSH/GSSG ratio. The GSH/GSSG ratio in the RRS is 3.7 versus 9.9 in the Organic Soy (an increase of ~270% in Organic Soy). The *in silico* predictions from the modified C1 metabolism model used in this study also was able to predict the loss of glutathione pool in the RRS. The model predicted a ~250% increase in the GSH/GSSG ratio in Organic Soy versus RRS, as shown in **Table 2**.

Table 1. Glutathione levels in the leaves of organic (glyphosate-sensitive) and RRS (glyphosate-resistant soy plants) [28].

Parameter	Organic	RRS
GSH/GSSG	9.9	3.7

Table 2. Comparison between *in vivo* and *in silico* results of GSH/GSSG ratio in organic soy (glyphosate-sensitive) versus RRS (glyphosate-resistant soy plants).

Experiment	Organic	RRS	Percent Change
			(Organic/RRS)*100
In Vivo	9.9	3.7	268%
In Silico	9.7	3.9	249%

5. Discussion

Mathematical modeling, in general, is highly dependent on many variables and assumptions. The models developed and integrated herein are based on literature aggregated based on specific criteria, from the known and accessible scientific literature. In this study, the critical assumptions are as follows:

1) All the reactions used in the models discussed occur in a single cell and at the cell surface;

2) The cell was assumed to be a well-mixed reactor with uniform concentration of a given biomolecular species in the volume of the cell;

3) All the simulations were performed over a continuous time period without considering the effect of environmental factors such as solar cycle, temperature, and soil condition, for example; and,

4) The results from this study are dependent on kinetic parameters and initial conditions of biomolecular species, information of which is based on the existing scientific literature.

The previous work performed by our group [4] indicated that genetic modification affected C1 metabolism via disrupting the formaldehyde detoxification pathway leading to depletion of glutathione. In this study, our purpose was to validate the depletion of glutathione. We focused our efforts on identifying experimental studies that have shown such disturbance in glutathione. As discussed earlier, Vivancos *et al.* observed a disruption in the glutathione pool in the glyphosate-treated glyphosate-resistant Soy [28]. By incorporating the glutathione synthesis and glyphosate catabolism in our previous *in silico* model of C1 metabolism, we were also able to predict similar decrease in the GSH/GSSG ratio in RRS, the glyphosate-treated glyphosate-resistant Soy.

Glutathione is the major source of non-protein thiols in most plant cells [30]. The chemical reactivity of the thiol group of glutathione makes it particularly suitable to serve a broad range of biochemical functions in all organisms. Highly reactive nature of glutathione along with the relative stability and high water solubility of GSH, makes it an ideal biochemical to protect plants against stress including oxidative stress, heavy metals, and certain exogenous and endogenous organic chemicals. Reduced GSH resulted in decreased plant growth. The plants also displayed reduced pigmentation due to low anthocyanin levels [31]. There is mounting evidence of GSH metabolism being affected by genetic modification; hence the ratio of GSH/GSSG is an ideal biomarker for biosafety analysis of GMOs [5]-[7].

Tausz *et al.* performed an in depth analysis of using glutathione levels as a marker of stress [21]. Their analysis revealed that stress conditions such as photo-chilling, photo-oxidative stress due higher salinity as well as drought conditions have a significant impact on the GSH/GSSG ratio. They identified a bi-phasic stress response in the form GSH/GSSG ratio in the plants exposed to the stress conditions. An initial phase that leads to an increase in GSH/GSSG ratio, possibly to compensate for the increased oxidative stress and a second, acclimation phase that results from prolonged exposure to the stressors. As we and others have reported genetic modification in Soy also increases the oxidative stress [4], and therefore it can likely be speculated that the oxidative stress caused by genetic modification may fall under the acclimation phase and can reduce the GSH/GSSG ratio. The results from this study are consistent with the GSH/GSSG ratio expected in acclimation phase, thereby providing validation for the expanded, integrative *in silico* model of C1 metabolism.

6. Conclusions

This research has demonstrated the viability of *in silico* modeling to predict observations from *in vitro* and *in vivo* web laboratory experiment. In this study, an *in silico* model of C1 metabolism was expanded to include glutathione biosynthesis and glyphosate catabolism. The simulations were conducted to predict the GSH/GSSG ratio for RRS as well as Organic Soy. These predictions matched very well with the *in vivo* measurement of GSH/GSSG ratio under the same conditions published by Vivancos *et al.* [28]. The concurrence of *in silico* and *in vivo* results shows that:

1) *In silico* analysis can be reliably used to understand complex molecular interactions in biological systems;

2) GSH/GSSG ratio is perturbed and is lower in RRS versus Organic Soy;

3) GSH/GSSG ratios can likely be useful as a reliable criterion for determining “substantial equivalence” of the RRS and Organic Soy, its non-GMO counterpart;

4) The RRS is not substantially equivalent to Organic Soy at the molecular level and calls into question the current practices of safety assessment of GMOs, particularly that used in allowing RRS Soy for public consumption; and,

5) These results indicate that a systems level understanding, as provided in our current and previous studies [4], can prove to be a synergistic method for biosafety assessment of genetically modified organisms.

Finally, this research further substantiates that our framework, developed herein, provides a modular platform for a "plug-and-play" type methodology for integrating molecular systems. In our previous work, systematic integration of C1 metabolism with oxidative stress was integrated with molecular mechanisms of genetic modification of Soy which induce oxidative stress. In this work we were able to expand such integration to include glutathione biosynthesis and glyphosate catabolism in a scalable manner. This scalability of our framework provides many new opportunities for continuing research in a transparent and collaborative manner.

7. Future Directions

The underlying meta-level parameters, such as nutritional value, composition, nutritional effects, metabolism, etc., used today, in determining substantial equivalence are historically and conceptually based on performance parameters used for medical devices and hardware systems, which may not meet the needs for assessing the equivalence of biological organisms, which are far more complex [8]. This research provides a systems-based approach to more rationally select criteria for assessing safety of GMOs.

The methodology and results of this effort can serve to motivate multiple areas of future research. One area, in particular, is using this expanded model to study the effect of glyphosate on the gut microbiome of animals including humans. Several recent analyses have shown that glyphosate residues have entered the food chain for human and animal consumption. Since C1 metabolism is an essential part of gut bacteria of animals, it is possible to predict effects of glyphosate on the glutathione pool in the microbiome, and its subsequent effects on various disease models affecting the health of the host.

Another area involves conducting multiple and randomized field trials from "off-the-shelf" products of RRS and Organic Soy to measure the GSH/GSSG ratios. Such efforts will help to understand if such differences exist in end-products and the scale of such differences.

Finally, we intend to make accessible the framework herein, using CytoSolve, to the broader GMO research community for open and collaborative research for assessing GMO safety. This open framework can enable researchers to test new data, molecular mechanisms, and hypotheses in the determination of GMO safety.

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Appendix A: List of Keywords

Keywords used for literature identification for glyphosate catabolism

1. Genetic modification oxidative stress signaling pathways
2. Glyphosate metabolism in soybean
3. Gyphosate AND hydrogen peroxide AND soybean
4. Metal factors regulating ascorbate peroxidase activity in plants AND iron
5. Kinetics of iron uptake in plants
6. Hydrogen peroxide and glutathione
7. Hydrogen peroxide and glutathione peroxidase in plants
8. Superoxide production AND photosynthesis
9. AMPA pathway
10. Antioxidant enzymes
11. Glutathione biosynthesis in plants
12. Glyphosate AND transgenic soybean AND formaldehyde AND AMPA
13. Formaldehyde dehydrogenase acting on lipid peroxide
14. Lipid peroxide as substrate for formaldehyde dehydrogenase
15. ROS AND catalase expression in plants
16. Glutathione peroxidase AND selenium AND plant
17. Requirement of GSH for formaldehyde dehydrogenase activity

Keywords used for literature identification for glutathione biosynthesis

18. Glutathione depletion
19. Glyphosate concentration in soybean plants
20. Kinetics of transport of glutathione between cytosol and chloroplast
21. GSH/GSSG ratio AND Transgenic AND Plants
22. GSH/GSSG ratio AND Organic AND Soy
23. Glyphosate AND degradation AND Kinetics
24. GSH biosynthesis AND Kinetics
25. Oxidized AND Reduced AND Glutathione

Appendix B

Table B1. List of parameters used *in silico* models of C1 metabolism integrated with glutathione biosynthesis and glyphosate cartabolism.

Kinetic Parameter	Description	Reference
kO_2^-	Rate constant for superoxide production	[32]
kmO_2^-	MichaelisMenten constant for superoxide production	[32]
kFe^3	"Rate constant for the conversion of superoxide to oxygen with simultaneous reduction of Fe^{3+} to Fe^{2+}	[33]
kH_2O_2	Rate constant for the production of hydrogen peroxide and oxygen from superoxide and H^+ (non-enzymatic)	[34]
kSOD	Rate constant for superoxide dismutase producing hydrogen peroxide from superoxide	[34]
KmH_2O_2	MichaelisMenten constant for catalase induced conversion of H_2O_2 to H_2O	[35]
kcata	Rate constant for catalase induced conversion of H_2O_2 to H_2O	[35]
kFe_1	Fenton reaction rate constant (hydrogen peroxide forming hydroxyl radical and anion with simultaneous conversion of Fe^{2+} to Fe^{3+})	[33]
kinitLR	Rate constant for lipid peroxidation reaction by hydroxyl radicals, forming lipid radicals	[36]
kLPO	Rate constant for the oxidation of lipid radicals	[36]
kLR1	Rate constant for the formation of L* and LOOH from LH and LOO*	[36]
$kLRFe_1$	Rate constant for Fe^{2+} induced formation of LO* from LOOH	[37]
$kLRFe_2$	Rate constant for Fe^{3+} induced formation of LOO* from LOOH	[37]
kfrLOO	Rate constant for LOO* fragmentation to alkane radical and aldehyde product	[38]
kFe_4	Rate constant for OH* induced formation of HO_2* from H_2O_2	[33]

Continued

kFe_5	Rate constant for Fe^{3+} induced formation of HO* from H_2O_2	[33]
kFe_8	Rate constant for H_2O_2 formation from HO_2*	[33]
kFe_9	Rate constant for the conversion of HO_2* and H_2O_2 to H_2O and OH*	[33]
kFe_6	Rate constant of Fe2+induced conversion of OH* to OH^-	[33]
kFe_7	Rate constant for the conversion of OH* and HO_2* to H_2O and O_2	[33]
kdH_2O	Dissociation rate of H_2O to H+ and OH-	[39]
KH_2O	Association rate of H^+ and OH- to H_2O	[39]
kAPX	Rate constant for APX induced conversion of Ascorbate to MDA	[34]
KAPX	MichaelisMenten constant for APX induced conversion of ASC to MDA	[34]
KAPXH	MichaelisMenten constant for APX induced conversion of H_2O_2 to H_2O	[34]
$k\_ASCH_2O_2$	Rate constant for ASC and H_2O_2	[34]
k_ASCO_2	Rate constant for superoxide reacting with ascorbate	[34]
kMDAR	Rate constant for molecular MDAR activity	[34]
KMDARM	MichaelisMenten constant of MDAR for MDA	[34]
KMDARN	MichaelisMenten constant of MDAR for NADPH	[34]
k_MDAMDA	Apparent rate constant of MDA	[34]
kDAR	Rate constant for molecular DAR activity	[34]
KDAR	MichaelisMenten constant of DAR for DHA	[34]
KDARG	MichaelisMenten constant of DAR for GSH	[34]
k_DHAGSH	Apparent rate constant of GSH and DHA	[34]
kGPxr	Rate constant of reduced GPx with H_2O_2	[40]
kGPxo	Rate constant of oxidized GPx with GSH to form intermediate GSGPx	[40]
kGSSG	Rate constant of GSGPx with GSH to recycle reduced Gpx	[40]
kGR	Rate constant for molecular GR activity	[34]
KGR	MichaelisMenten constant of GR for GSSG	[34]
KGRN	MichaelisMenten constant of GR for NADPH	[34]
kNAP	Rate constant for the conversion of NADP to NADPH	[34]
VCPLG	Conversion of glyphosate to sarcosine	[41]
Kglypexud	Extrudation of Glyphosate	[42]
KCPLG	MichaelisMenten constant for the conversion of glyphosate to sarcosine	[41]
KSOX	Catalytic rate constant for the conversion of sarcosine to glycine	[43]
kSOX	MichaelisMenten constant for the conversion of sarcosine to glycine	[43]
KmGO	MichaelisMenten constant for the conversion of glyphosate to AMPA	[24]
kGO	Catalytic rate constant for the conversion of of glyphosate to AMPA	[24]
kAMPA	Rate constant for the conversion of AMPA to methylamine	[44]
VMDH	Maximal production of formaldehyde from methylamine	[45]
KMDH	MichaelisMenten constant for the formation of formaldehyde from methylamine	[45]