Comparative Study on Safety Protocols of GM Foods approved in developed and developing countries, and possibilities for Bangladesh

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Bachelor of Science in Biotechnology

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Declaration

It is hereby declared that:

1. The Thesis report submitted is our own original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Approval

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Abstract

A Genetically Modified Organism (GMO) has had its DNA altered via modern biotechnology. GM Food is one of the sources to ensure food security for the future, to escape the risk of malnutrition and starvation. Food safety regulations declare any GM food item safe for consumption. The process often references multiple dossiers, following the Codex Alimentarius, the FDA protocols, etc. However, comparative studies for reflecting regulatory practices in context to GM food approval are sparse. The aim of this thesis is to find a clear harmonization between two such safety protocols, one from the USA as a developed country and another from the Philippines as a developing country, by comparatively assessing each of their approval processes for different GM foods with nutritional benefit, which may then be used to discover an applicable regulatory process that may be implemented in Bangladesh. In this thesis comparative analysis between the core information and specific information for Anthocyanin tomatoes from the USA and Golden rice from the Philippines is done, then in accordance with the international standards set by the Codex Alimentarius studied to identify overlaps found with both cases. Identification of where these overlaps also follow the specifications of Bangladeshi guidelines is used to allow for possible harmonization of all three protocols. In light of this information, steps are suggested for the possible adaptation of these processes for the possible approval of GM food in Bangladesh through a harmonized protocol.

Keywords: Codex Alimentarius; Food Safety; Harmonization; Comparative Analysis; Anthocyanin Tomatoes; Golden Rice

Dedication:

We dedicate this thesis to our supervisor for guiding and supporting us during this one year long thesis. Our families for their love and support during our undergraduate years, as well as all the friends we made along the way and the ones who stayed for the longest time.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

- BFSA Bangladesh's Food Safety Authority
- BSTI Bangladesh Standards and Testing Institute
- CFSAN Center of Food Safety and Applied Nutrition
- FAO Food Agriculture Organisation
- FDA Food and Drug Administration
- FD&C Act Federal Food, Drug and Cosmetic Act
- GE Genetically Engineered
- GHI Global Hunger Index
- GMO/GM Genetically Modified Organism / Genetically Modified
- IRRI International Rice Research Institute
- NHANES National Health and Nutrition Examination Survey
- NPS Norfolk Plant Science
- OECD Organisation for Economic Co-operation and Development
- ORF Open Read Frame
- r-DNA Recombinant DNA
- SGF Simulated Gastric Fluid
- T-DNA Transfer DNA
- UN United Nations
- VAD Vitamin-A deficiency
- WTO World Trade Organization

Chapter 1: Introduction

1.1 Overview

A genetically modified organism (GMO) is a plant, animal, or microorganism whose genetic material has been altered through techniques of genetic engineering, often involving the transfer of specific genes between organisms that might not be related to each other. GM food has become the common term consumers and popular media use to describe foods that have been created through use of genetically engineered (GE) organisms. This term is not usually used for referring to plants or animals developed with selective breeding, an agricultural technique that has been for centuries to achieve desirable traits from crops and animals by breeding and selection of traits over multiple generations.

Direct genetic modification is a relatively recent invention, discovered as early as the 1980s, which has been rising in popularity as an invaluable tool in many fields of bioengineering, not least of all the development of GMO Crops. Early methods of genetic modification were considered too costly to pursue, either in terms of economical expenses due to inefficient methodology, or due to poor public reception in a more metaphorical context, rising from misinformation and controversy surrounding an emergent field of scientific innovation. However, numerous concerns surrounding this have been addressed and resolved with the creation of simpler, more accessible and precise methods of genetic engineering and the development of thorough biosafety assessment protocols. For example, CRISPR-Cas9 has become the most popular choice of GM methods, providing a cheap and safe way to create more predictable and precise changes, often eliminating the need for introducing new genes from unrelated species. As a result, older transgenic methods are being phased out more quickly in favor of CRISPR-Cas9. The main hurdles faced by modern genetic engineering are the hyper-strict regulations and negative public perception, all of which contribute to consumer unease and uncertainty about the future (Teferra, 2021). To address this, many regulatory practices have been adopted on international and many national levels which allow for safer and reliable modifications and usage of these GMO products, such as The Codex Alimentarius (Codex Alimentarius, 2003); Bangladesh in particular has adopted the “Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants” document on the national level (Bangladesh Standards and Testing Institute [BSTI], n.d.).

With the increased and somewhat normalised usage of GMO as of recent years, there is now a greater pressure for such crops and food sources to meet nutritional requirements than ever before. With population increasing worldwide as well as scarcity of various resources will put pressure on agricultural work sectors in the upcoming years to not only provide food in ample quantities and nutrition but also within lesser resources than what is available at present. It is estimated that at least one billion people worldwide experience starvation and two billion people are affected by one or more micronutrient deficiencies, especially vitamin A, iodine and iron, which is often referred to as hidden hunger (Jacobsen et al., 2013).

As of 2024, Bangladesh is ranked 84th out of 127 countries on the Global Hunger Index (GHI), with a score of 19.4, signifying a moderate level of hunger. The GHI score is derived from four indicators, namely malnutrition, child stunting, wasting, and mortality. According to GHI, child stunting and child wasting refers to the lower than expected growth of children under the age of five, specifically the lower height i.e. stunting and weight i.e. wasting of the children, which are considered extreme signs of undernourishment in the population. The statistics from GHI show that 11% of children under the age of five are wasting and 23.6% of children under the same age group are also stunted, this indicates that a significant number of the children in Bangladesh are severely undernourished (Index, 2024).

Therefore, the purpose of this study is to compare the steps taken in food safety protocols for the case of foods derived from GMO with nutritional benefits, especially surrounding its approval in developed and developing countries, and also to observe for harmonization and possibilities of learning of similarity in Bangladesh.

1.2 Background Information

1.2.1 Justification of Regulations Surrounding GMO

In typical productions of GMOs, some unintended side effects do become present for a transfer DNA (T-DNA) insertions, especially common for genes where in source organisms they offered positive and negative regulatory actions (i.e. genes were responsible for up regulation and down regulations of certain genes). This unpredictability is mostly due to how the T-DNA may interact in the recipient organism, as the pre-mRNA splicing may change the intended amino acid

sequence of the T-DNA if the insertion area gets spliced or altered further during gene translation during expression. Furthermore, the effect of a T-DNA in the regulatory region of a protein coding gene for genetic enhancement of its expression may be more complicated than direct protein genetic modification, as many other factors may be involved in the upregulation or downregulation of a protein's expression on the genetic level (Ladics et al., 2015).

One example being that in early attempts of creating a tomato fruit (*Lycopersicon esculentum*) with upregulated anthocyanin expression, a regulating gene from maize (*Zea mays*) was attempted to be adopted in hopes for increasing the flavanol and anthocyanin content in the fruit. It was found that the transformed fruit had some elevated flavanol content but the anthocyanin content was unchanged when compared to the un-transformed tomato fruit. This was concluded to be the case because these GMO tomatoes lacked the other genes necessary for anthocyanin up regulation that maize had. Thus this GMO tomato was never approved for usage, as its intended change was not found in the fruit, an "elite" event was not seen (Ladics et al., 2015). In similar circumstances, for Golden Rice 1 (SGR1) the base plant (*Oryza Sativa*) was transformed with *psy* gene from Daffodil (*Narcissus Pseudonarcissus*), but it only produced 1.6 μg/g of beta-carotene under greenhouse conditions; this was not the "elite" event the project aimed for. Golden Rice 2 (GR2E) that was developed in 2005 by Syngenta, aimed to increase provitamin-A production by replacing the *psy* gene from Daffodil with the *Zmpsy1* from Maize and the *crt1* gene from *Pantoea ananatis*, with the capacity to favorably accumulate up to 37 μg/g of beta-carotene in the grains, proving to be the "elite" event that was desired for Golden Rice (Paine et al., 2005).

"Elite" event/s in terms of genetic modification refer to creation of top performing organisms with the desired traits that are either novel from the common/recipient strain or are enhanced in comparison to that of the recipient's performance. The goal in the end remains similar to what traditional breeding aims to accomplish, products with better or improved traits. In GMO crop creation, there are extensive vetting processes that allow elimination of any transformed plants which fail to show the desired changes, as seen for the maize gene inserted tomato fruits (Ladics et al., 2015). This practice of vetting for modified traits of GMOs in development follows what

the Codex Alimentarius suggests should be done for gaining the approval of the development and usage of such crops.

The Codex Alimentarius (CAC/GL 45-2003, Section 4 (point 44) and Annex ii (point 10)) describes that GMO plants in their key component wise comparison to the native/non-gmo plant, should have similar level of nutrient at least, but if modified for higher level of nutrient then it is ideal that the GMO would have nutrient/s level that is closest to upper level of intake for those nutrients based on history of safe use and published data.(Codex Alimentarius, 2003)

The most important criteria in the GMO product development process is that of the efficacy or the performance relating to the transformation i.e. does the trait bring about the desired phenotype, meeting the product specifications. For GM crops, the next important criterion for identifying the lead events is the **molecular characteristics** of the event. The desired, inserted gene must be present in a single locus as a singular copy of that gene. The insertion should not include vector backbone nor disrupt function or sequence of native protein of the organism or even form a chimeric novel protein. Minimal locus rearrangement should be observed, however the integrity of the gene cassette should be preserved. Although none of these parameters have been demonstrated to actually influence the safety of the crop, the importance given in consideration of these parameters is based on hypothetical, minimal-possibilities. Due to most products requiring multiple approvals, the requirements from firm jurisdictions governing the event selection criteria (Ladics et al., 2015).

When these elite event/s are identified, extensive safety assessments are conducted which also include studies on safety of the new expression/s of the protein, molecular characterization of inserted genes and the impact of the insert on overall crop performance and composition.

1.2.2 Prevalences of GMO Food in Agriculture

In the USA, the large-scale adoption of GM crops began in 1996 and have since spread extensively in the country's agricultural practices. According to Jacobsen et al (2013), GM crops make up for 73% maize, 87% cotton and 91% of soybean; and the limiting factor for soybean at one point was the availability of its seeds, the glyphosate resistant (Roundup®) soybeans. While

many farmers of the USA have always been accepting of GMO, the acceptance of GMO in Europe has been slow due to the severe public opposition to it. To the point that in the EU only 114,500 ha of arable land was growing Bt maize as the only GM crop, mostly in Spain, which is estimated to be about 0.01 % of the available European agricultural area (Jacobsen et al., 2013).

Despite the evident fears in many countries still involved, GM food can allow for many opportunities. Bt brinjal, an indian-produced GM crop that is the first approved GM crop of Bangladesh, is an important crop for the future of biotechnology. The success of this initial crop has paved the way for further developments in the research surrounding this crop. Fortunately, Bt brinjal has had positive beginnings, showing a steady increase in yearly adoption and noteworthy socioeconomic benefits in Bangladesh and in other countries that have also adopted its variants (Shelton et al., 2018). Thus for successful adoption of GM foods there are factors such as the public perception of how the crops can impact in terms of the risks and the benefits of GM acceptance, as well as particular country's participation in international environmental agreements and their agroindustry's supports in the adoption of the modified crops (Jacobsen et al., 2013).

1.2.3 Nutritional GMO Concepts expanded

GMO that has been developed for addition or enhancement of nutritional value is often referred to as nutritional GMO. Nutritional GM foods are still new in terms of human consumption and only few countries have them actively in their food markets, USA being one of them (Abrams et al., 2023), (Nishtar et al., 2024). Most GM food crop development of this category aims for enhancing the nutrient level that might be low in the original crop plant (Butelli et al., 2008), or adding new genes for introducing nutrients that might have not existed in the original crop (Oliva et al., 2020). Nutritional GM food usage is still under much scrutiny, especially due to sparse research for approvals despite developments of these crops show the benefits, adding on to which because of lack of adequate data on the long term effects of human health still makes these crops relatively new in the sphere of human consumption and lacking supporting evidences on actual benefits in prolonged consumption (Abrams et al., 2023), (Nishtar et al., 2024). However, research done so far does prove that crops that have been specifically modified for increased nutrients show the promised expressions of the trait within the limits that are possible for human

consumption, but the question remains for the aid it may give as a food product (Nishtar et al., 2024).

1.3 Objective of Study

The main objective of the study is to analyze food safety protocols regarding the safety of foods that are from genetically modified specifically for nutritional benefits by case study based analysis from a developed country i.e. the USA and a developing country i.e. the Philippines. Furthermore, possibilities of harmonization of protocols will be explored by comparison with regulatory practices of Bangladesh based around GM food. However, a few more objectives have been aimed to be achieved.

1.4 Specific Aims

- ➔ analyze and compile the available food safety data regarding the safety of GM food in context of their molecular characterizations.

- ➔ observing and comparing regulatory practices that are involved in the approval of such crops, by using case studies as models: the USA model as a developed country and the Philippines model as a developing country.

- ➔ possibilities of harmonization of protocols will be explored by comparison with regulatory practices of Bangladesh based around GM food.

Chapter 2: Materials and Methodology

2.1 Material:

This study is comparative in nature, meaning that this study will take reference from safety assessment dossiers and comment on the nutritional crops that are approved in the USA and Philippines. Dossiers relevant for the developed country case i.e. USA, were available through the website of the U.S. Food and Drug Administration (FDA) (U.S. Food and Drug Administration [FDA], n.d.). The dossiers that were necessary for the safety assessment done in the Philippines i.e. the developing country case were not available completely, thus secondary sources like review papers were taken as reference that were followed for the approval in there (Oliva et al., 2020). Along with that, for the purpose of comparing with practices in Bangladesh, reference will be made to how GM food may get approved in Bangladesh in accordance to "Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants" adopted as standard by Bangladesh Standards and Testing Institute (BSTI) (Bangladesh Standards and Testing Institute [BSTI], n.d.). For international food safety regulations the Codex Alimentarius will be referred to (Codex Alimentarius, 2003). The main idea of focus will be on the core information that is required to be investigated for these dossiers, specifically the molecular characterisations of the newly introduced traits of the GM food crops. The following case studies from the aforementioned country models i.e. USA and Philippines will be elaborated below with how Bangladesh emphasizes GM food safety assessment in their own documents.

2.1.1 A Case from a Developed Country: United States of America for Anthocyanin Tomato

Anthocyanin tomato, more commercially known as Purple Tomato, is a GMO crop specifically developed to have increased anthocyanin content compared to commercially available variants of tomatoes. It was developed in the United Kingdom (UK) under the company Norfolk Plant Science (NPS), a company that was derived from John Innes Centre and The Sainsbury Laboratory (*Norfolk Plant Sciences | John Innes Centre*, n.d.).

Even though Anthocyanin Tomato was developed in the UK, it is also approved for use in the USA. The Food and Drug Administration (FDA) of the USA is a science based agency for public health and regulatory actions necessary around approval and maintenance of safety surrounding food and other consumable products in the USA (U.S. Food and Drug Administration [FDA], n.d.). Center of Food Safety and Applied Nutrition (CFSAN) is a branch of FDA that is mainly

focused for this purpose and conducts the regulatory actions and the research that gets involved with it. As USA follows their own unique food safety framework, their workflow will allow comparison with the other countries being discussed in this thesis i.e. Philippines and Bangladesh. The CFSAN of FDA did the food safety assessment necessary to approve Anthocyanin Tomato in the USA for human consumption and they deemed it safe for humans. (U.S. Food and Drug Administration [FDA], 2023). The framework that CFSAN of FDA follows allows the USA to be more liberal in accepting more beneficial, well-performing GM crops into their farming practices.

2.1.2 A Case from a Developing Country: The Philippines for Golden Rice

Golden Rice is a GM crop developed to address the problem of Vitamin-A deficiency (VAD), which is a distressingly common health disorder in developing countries. The development of Golden Rice began in 1993, led by scientists Ingo Potrykus and Peter Beyer, who sought to provide a sustainable solution to VAD through the use of biotechnology. The GMO crop was initially developed in collaboration with the International Rice Research Institute (IRRI), assisted by the Rockefeller Foundation, the European Union and the Swiss Federal Office for Education and Science (Beyer et al., 2002).

Although it began development in the University of Freiburg in Germany, Golden Rice was soon approved for use in the Philippines. On 29th of October in 2020, an application for the commercial propagation of Golden Rice was submitted to Philippine government regulators. On 21st July, 2021, the Director of the Philippines Department of Agriculture's Bureau of Plant Industries (DA-BPI) signed off on the Commercial Propagation Permit for Golden Rice in the Philippines, making Philippines the first country to approve nutrient-enriched Golden Rice for planting. (https://www.goldenrice.org/Content2-How/how4_regul.php)

2.1.3 A Case Yet to be Made: Bangladesh

Bangladesh has yet to approve any nutritional GMO as safe for consumption in the country. For example the approval for Golden Rice has been pending in Bangladesh since 2017 (*Golden Rice*, 2023). An effective comparison between Bangladesh's "Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants", the published dossiers for the Anthocyanin

Tomato and Golden Rice, and the contextual information available from The Codex Alimentarius can help to understand safety assessments and perhaps if nutritional GM food can possible ever get approved in Bangladesh.

2.2 Methodology

The methodology by Mimmi (2021) is being referred to in this thesis for the comparison of the core information focus of the molecular characterisations of the case mentioned nutritional GM food for the USA and the Philippines and also for comparing that to Bangladesh's food safety assessment guidelines. Furthermore, the contextual interpretation that was made by each country i.e. Bangladesh, the Philippines and the USA of the international protocols, The usage of The Codex Alimentarius will also be commented on and judged to confirm the possibility of harmonizing the protocols. The following chart (figure 2.1, next page) outlines the methodology utilized for the comparative study conducted in this paper:

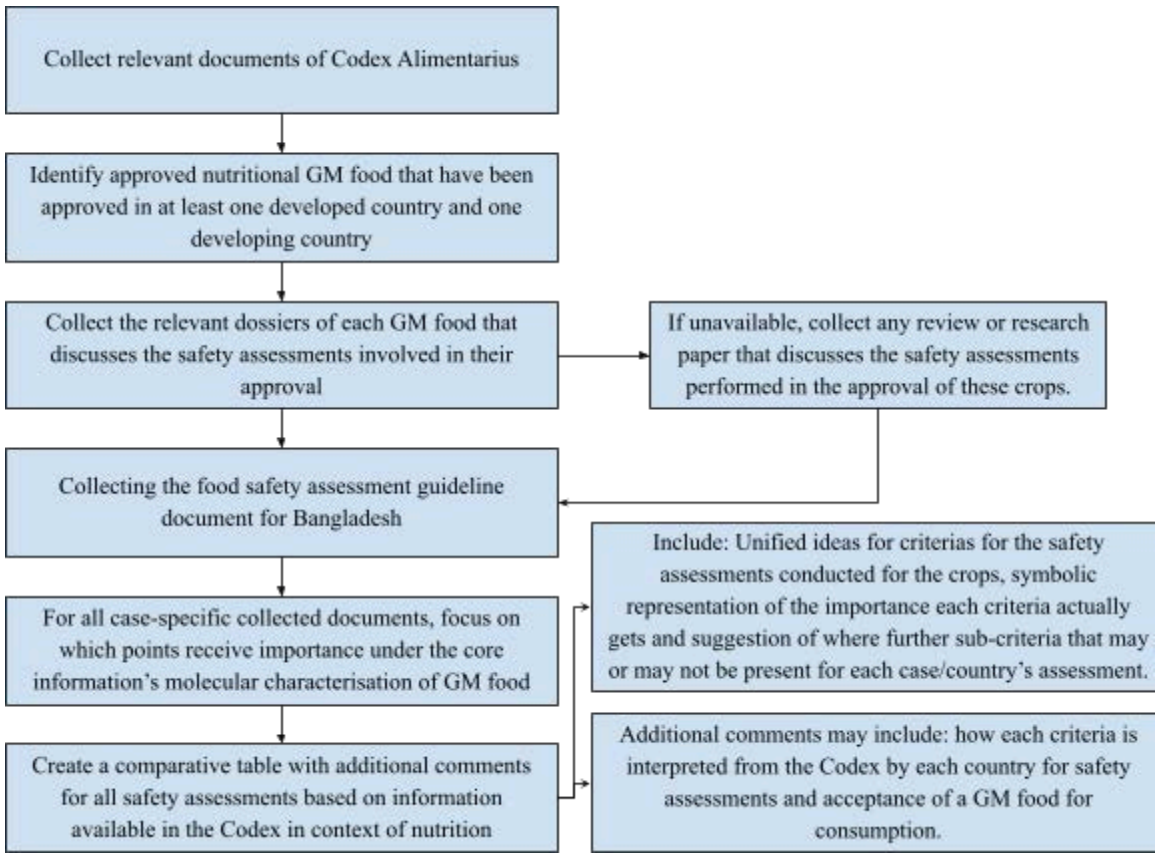

Figure 2.1: *Workflow of the Methodology followed used for this thesis*

Chapter 3: Results

Molecular Characterization of GMOs may be defined as the usage of identifying genetic markers to determine the genomic characteristics of a GMO that make it distinct from its unmodified counterpart. During a food safety assessment, Molecular Characterization is utilized to determine the success of the desired transformation and confirm the safety of the product in terms of genetic attributions and assay evidence as well as the absence of undesirable traits. In the following chapter, we shall elaborate on and explain 7 key tests performed via Molecular Characterization and food safety assessments to assess the safety of Golden Rice and Anthocyanin Tomatoes as GM Food. They are:

1) The history behind the breeding and selection of successful cultivar.

2) Confirming the successful transformation of the GMO with the desired traits.

3) Ensuring the absence of dangerous elements and modifications.

4) Verifying the inheritance stability of the transformation.

5) Analysis of all Open Read Frames.

6) Searching for the presence of unwanted proteins.

7) Assessing the effect on humans and human diet.

3.1 Molecular Characterization: the GM Foods' Identity from the Core Information

In this chapter, Molecular Assessment is used to prove the successful transformation and genetic stability of Anthocyanin Tomatoes and Golden Rice. The results of their food safety assessments are comparatively assessed by summarizing their individual case dossiers and comparing them to both the Codex Alimentarius and Bangladeshi food regulation. (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex Alimentarius, 2003), (Oliva et al., 2020), (U.S. Food and Drug Administration [FDA], 2023)

In this regard, both Anthocyanin Tomatoes and Golden Rice are ideal candidates, as they have each individually undergone the relevant food safety assessments in two separate countries, USA and Philippines respectively, and have been declared safe for human consumption and usage (U.S. Food and Drug Administration [FDA], 2023). Thereby, making these two cases suitable choices to compare with the 'Case yet to be made' for Bangladesh. Any possible difference in protocols for each country may be irrelevant, since the food safety assessment protocols for all three countries follow the Codex.

The anthocyanin tomatoes were modified to elevate anthocyanin expression, a naturally occurring plant phenol with antioxidant and anti-inflammatory properties. It's linked to preventing diseases such as cardiovascular issues, diabetes, obesity, and cancer, and may provide neuroprotective effects, as evidenced by animal studies. This prompted extensive research on enhancing their levels in food crops.(Colanero et al., 2020). Tomatoes (*Solanum lycopersicum*), among the most consumed vegetables globally, have been targeted for genetic modification to enhance anthocyanin levels, as it naturally contains less due to an incompletely activated flavonoid pathway (Colanero et al., 2020).

Anthocyanin Tomato, also known as Purple Tomatoes, are the successful GM crop with increased anthocyanin content compared to the other commercial tomato variants (Butelli et al., 2008). The modification to the biosynthetic pathway responsible for anthocyanin production in these tomatoes was performed with two transcription factor-encoding genes, Delila (*Del*) and Rosea 1 (*Ros1*), obtained from *Antirrhinum Majus*, the Common Snapdragon plant. The modifications of the tomato plants were expressed using their fruit-specific E8 promoter that was put together with the recombinant binary plasmid pDELROS, delivered via *Agrobacterium tumefaciens* (strain LBA4404) through triparental mating. This resulted in selecting crossbred tomatoes that are homozygous for the intense purple pigmentation from the high anthocyanin content in both the peel and flesh (Butelli et al., 2008).

Golden Rice was designed to be rich in Beta-carotene, a provitamin-A carotenoid, which could then be used by the body to synthesize Vitamin A. Golden Rice is designed to augment the diet of people from poorer countries, the majority of whom are at subclinical level deficiency in

vitamin A. A common misconception is that Golden Rice must replace the need for Vitamin-A outright; it does not, as the majority of its target demographic are deficient and can benefit immensely from even a minor modification to their provitamin-A intake. The key genetic modification of GR involves the introduction of two genes: *Zmpsy1* (Zeamaize Phytoene Synthase 1) from Maize (*Zea Mays*) and crt1 (Phytoene Desaturase) from the bacterium *Erwinia Uredovora*, which are used to greatly increase the amount of beta-carotene produced and deposited in rice endosperm; this is also what gives GR its distinctive golden color (Beyer et al., 2002).

In 2005 Syngenta successfully created a variant of Golden Rice, Golden Rice 2 (GR2E), that produced approximately 23 times more beta-carotene than its predecessor (SGR1). This was possible by using *Agrobacterium* (*A. tumefaciens*) mediated transformation of the japonica rice cultivar Kaybonnet with the transformation vector pSYN12424 and gene insertion of the *Zmpsy1*, *SSU-crt1* and *pmi* genes (Paine et al., 2005).

In table 3.1 are listed the abbreviated phrases that will be used in this chapter, to visually signify the relation between each case and dossier, along with identifying how closely each of them actually follows the Codex Alimentarius.

Table 3.1: *Abbreviations used for making comparison-based commentary*

Full Form	**Abbreviation**	**Description**
Specially Mentioned	S.M.	Points that have been emphasized in the dossier they way it has been in the Codex
Specially Mentioned in Details	S.M.D.	Points where particular subcategories have been expanded upon for certain cases
Partially Mentioned	P.M.	Points that have been emphasized in the dossier they way it has been in the Codex
Not Mentioned	N.M.	The dossier does not mention the information in any way the Codex do

3.1.1 Breeding and Selection

Breeding and selection is still a process that gets integrated into the GM production more so for plants. This is to ensure that the GM plants that get produced have traits that are possible to be passed along to plants of the same species to avoid further need for recombinant DNA (r-DNA) technology for creating more varied crops. This also ensures that the crops can be more readily available and possibly help to reduce the price of seeds for farming. However for consumers it could raise the question of non-GM plants that get marketed for their native traits also carrying these modified traits due to cross-pollination, further research will be necessary to assess the safety in those particular cases (Jacobsen et al., 2013). For the sake of this paper, the gm plants of Anthocyanin Tomatoes and Golden Rice were deemed safe for their own usage within their tested varieties. (Oliva et al., 2020), (U.S. Food and Drug Administration [FDA], 2023).

For anthocyanin tomatoes:

The modification to the biosynthetic pathway responsible for Anthocyanin production in these tomatoes was performed with two transcription factor-encoding genes, Delila (Del) and Rosea 1 (Ros1), obtained from *Antirrhinum Majus*, the Common Snapdragon plant. The modified tomato plants were then expressed in the Cultivar MicroTom, with the aid of a specific promoter, the fruit specific E8 promoter from tomatoes, these transcription factors were placed together with the promoter in a recombinant binary plasmid (named pDELROS) which got carried to MicroTom tomatoes via *Agrobacterium tumefaciens* (strain LBA4404, use of triparental mating for this) for transformation of the tomatoes. Repeated selection of transformed tomatoes for cross breeding resulted in tomato fruits with intense purple coloration due to its Anthocyanin-rich nature; a pigmentation that was spread throughout the plant, both in its peel and flesh. This line of rich purple colored anthocyanin tomatoes was in the research called “homozygous T2 *Del/Ros1-N* ” (Butelli et al., 2008). The repeated selection involved the selection and breeding of transformed tomato plants exhibiting optimal expression of the engineered trait. NPS transformed tomato plants (*Solanum lycopersicum cv.* MicroTom) and identified the primary transformant with the highest anthocyanin accumulation (designated as *Del/Ros1-N*) to develop a homozygous *Del/Ros1-N* trait within both the MicroTom and MoneyMaker genetic backgrounds through selective breeding. NPS reported that, to produce *Del/Ros1-N* in MoneyMaker, they crossed T1 (transformed generation 1) *Del/Ros1-N* MicroTom plants with

MoneyMaker to create a purple fruited F1 population. One fruit from this F1 population was self-fertilized, resulting in a dark purple F2 fruit, which was subsequently self-fertilized and propagated for eight generations from a single seed to establish the *Del/Ros1-N* stockline in MoneyMaker. A similar approach was employed using the T1 plants for MicroTom, but only for six generations, to develop the *Del/Ros1-N* stockline in MicroTom. (U.S. Food and Drug Administration [FDA], 2023). This proved in the original research as well as the regulatory protocol practice that the modified gene is not only transferable to another plant (i.e. Money Maker) through cross pollination but can stay present in the particular plant through several generations only if selective breeding is done correctly to preserve that trait. Figure 3.2 illustrates the summarised process of creation of the transformed *Del/Ros1-N* stocklines of the two tomato cultivars.

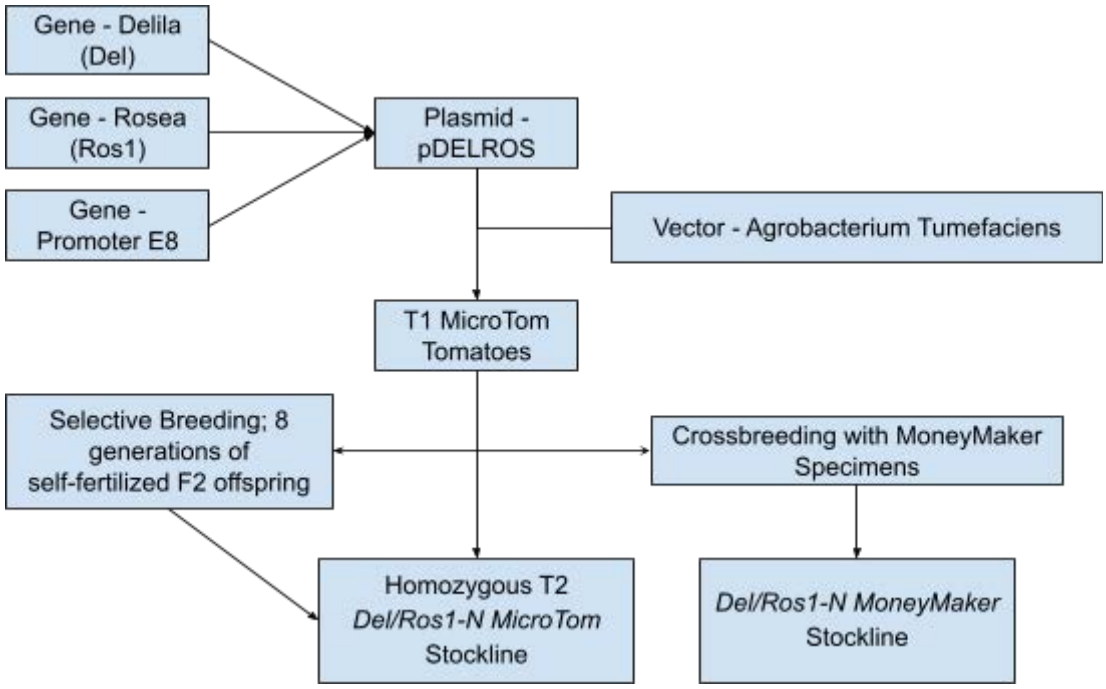

Figure 3.2: *Creation of transformed Del/Ros1-N Stockline of MicroTom and MoneyMaker Cultivars*

For golden rice:

For Golden Rice, the breeding and selection process is elaborated properly in the inheritance stability section (3.1.4). The modification to the biosynthetic pathway responsible for GR2E was

performed by transforming the embryogenic calli of the japonica rice cultivar Kaybonnet with a vector plasmid (named pSYN12424) by way of *Agrobacterium tumefaciens*-mediated transformation. The goal of this endeavor was to modify the biosynthetic pathway of *Oryza Sativa* to allow the production of various provitamin-A carotenoids, primarily beta-carotene ("Biotechnology Notification File No. 000158," 2018). In pursuit of this goal, IRRI utilized the *Zmpsy1* gene from *Zea mays* (Phytoene Synthase) and the *crtI* gene from *Pantoea ananatis* (Phytoene Desaturase). These enzymes enable a new metabolic route for the conversion of Geranylgeranyl Diphosphate into Lycopene, which is the precursor to the provitamin A carotenoids, alpha- and beta-carotene. The *pmi* gene from *Escherichia coli* (Phosphomannose Isomerase) was used in tandem with the Selective Media Mannose to filter for and identify viable transformants, since Mannose cannot normally be utilized by plants as a source of nutrition. The transformed calli were then transferred to a plant regeneration media for propagation. A transformed plant (T0) containing the GR2E event was subsequently chosen and crossed into the following indica rice cultivars: PSB Rc82, BRRI dhan 29 and IR64, for molecular characterization and compositional analysis (Oliva et al., 2020). Figure 3.3 illustrates the creation process for GR2E.

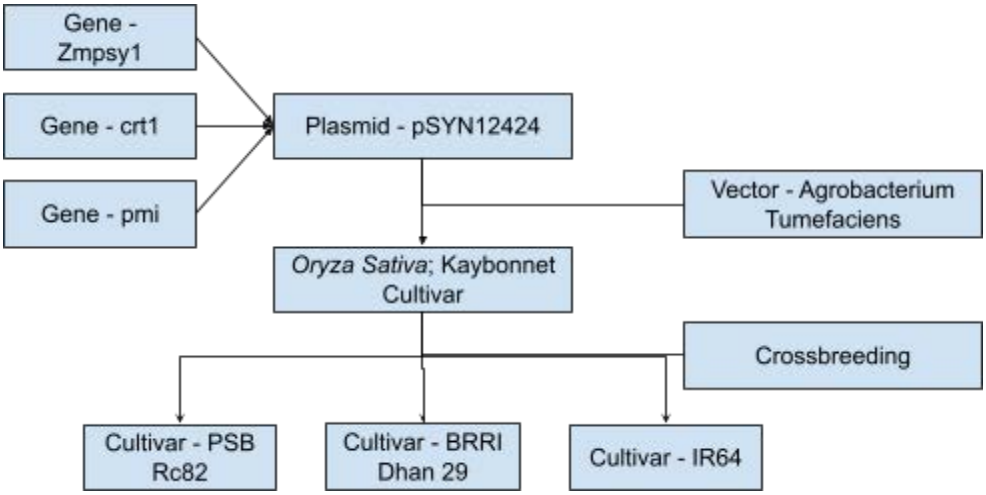

Figure 3.3: *Creation of GR2E Stocklines*

What does Codex say?

Due to the unpredictability of the r-DNA insertion in the gm plant, breeding of plant with non-GMO may cause unintended/unpredictable changes, according to point 14 from section 3,

and this needs to be accounted for by rejecting the undesirable offsprings of the breeding process through selection of the best phenotypic plants (Codex, 2003)

Guideline for food safety assessment in Bangladesh

The chapter 6 section 4 that mostly mentions for nutritional modifications in the guideline of Bangladesh (Bangladesh Standards and Testing Institute [BSTI], n.d.), also mentions the usage of breeding specifically for desired traits even for nutrient level elevation, however breeding and selection alone can lead to changes that are more broad and unpredictable than r-DNA based modifications i.e. nutritional gm crops.

Comparison for Harmony of Protocols

Table 3.2 comparatively illustrates and summarizes the similarities and differences between the regulatory practices which were followed for the approval of both Anthocyanin Tomatoes and Golden Rice, in the USA and Philippines respectively, in regards to their relevant Breeding and Selection associated information. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003), (Oliva et al., 2020).

Table 3.2: *Comparison of Breeding and Selection Data*

Breeding and selection information required	**Anthocyanin Tomatoes (USA)**	**Golden Rice (Philippines)**	**Bangladesh Guidelines**
Plants that were bred for specific traits provide the desired offsprings	S.M.	P.M.	S.M.
Application of traditional selective breeding for trait amplification	S.M.	P.M.	S.M.
Description of the phenotypic traits aimed for selective breeding	S.M.	P.M.	S.M.

S.M. - Specially Mentioned, P.M. - Partially Mentioned.

3.1.2 Confirmation of Intended Genetic Change

This kind of confirmation is to ensure that the changes made are not only phenotypically present but also are present on the genetic level. The GM plants that get selected with the desired phenotypes are usually assessed for the genetic changes to ensure that the desired trait is indeed due to the intentional modifications made to the genome of the crop plant (Butelli et al., 2008).

For anthocyanin tomatoes:

According to the FDA report (U.S. Food and Drug Administration [FDA], 2023), NPS used quantitative PCR (qPCR) and Southern Blot Analysis for confirmation analysis of single DNA insertion in both the stockline tomato. "Using high throughput sequencing, NPS amplified the *Del* and *Ros1* genes in a *Del/Ros1*-N MicroTom tomato and confirmed the integrity of the insertion. NPS used inverse PCR to assess the T-DNA flanking sequences and found that there is a 94 bp deletion in the tomato genome at the insertion site, a 52 bp deletion at the T-DNA right border (RB) region and a 75 bp deletion at the T-DNA left border (LB) region.". Figure 3.4 shows the flow of work followed for molecular confirmation of genetic changes for anthocyanin tomatoes.

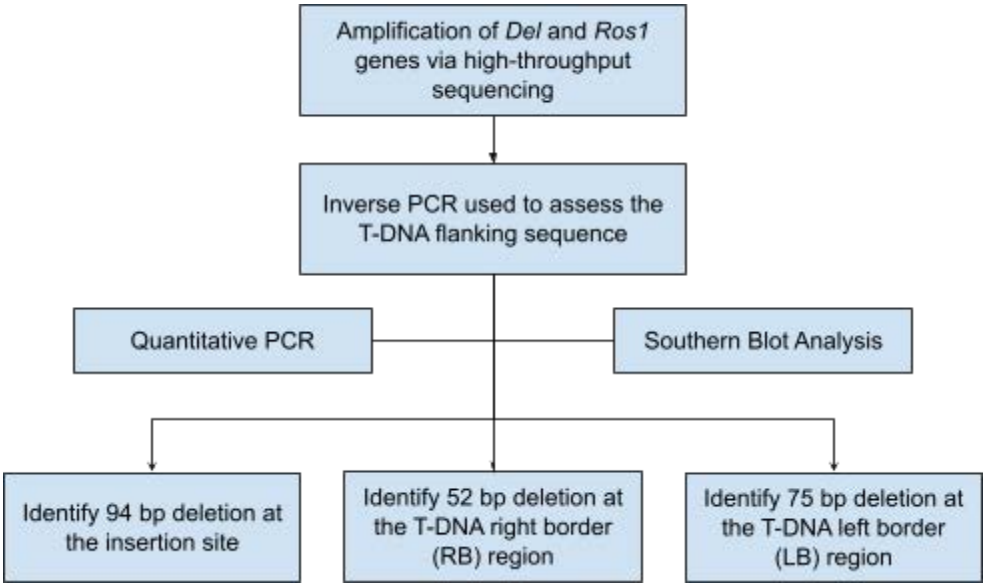

Figure 3.4: *Molecular assessment for the confirmations of genetic changes in anthocyanin tomatoes*

For golden rice:

IRRI confirmed the insertion event in GR2E rice cultivars via Southern blot analysis and direct sequencing of the PCR products that were extracted from the insertion site and amplified. The *HindIII* and *SphI* enzymes, both containing unique restriction sites within the T-DNA of pSYN12424, were used to determine the copy number of the introduced DNA within the GR2E genome. Hybridization of *HindIII*-digested genomic DNA from homozygous plants of GR2E in four genetic backgrounds (Kaybonnet, PSBRc82, BRRI dhan29 and IR64) with the *Zmpsy1* or *pmi* probes resulted in the detection of a single fragment of 7900 bp. Hybridization of the T-DNA with the probe specific for *SSU-crtI* yielded a single 7200 bp fragment. Hybridization of *SphI*-digested GR2E genomic DNA with the *Zmpsy1* or *SSU-crtI* probes yielded a single fragment of 6900 bp and upon hybridization with the *pmi* probe yielded a single fragment of 5500 bp. Therefore IRRI concluded that GR2E rice contains a single, intact DNA insertion that is identical to the T-DNA region of plasmid pSYN12424, with the exception of small deletions at the ends of both the right and left borders, which were negligible. The two deletions do not affect the integrity of the expression cassettes containing the genes to be transformed (Oliva et al., 2020). Figure 3.5 shows the steps for the molecular confirmation for genetic changes done for GR2E.

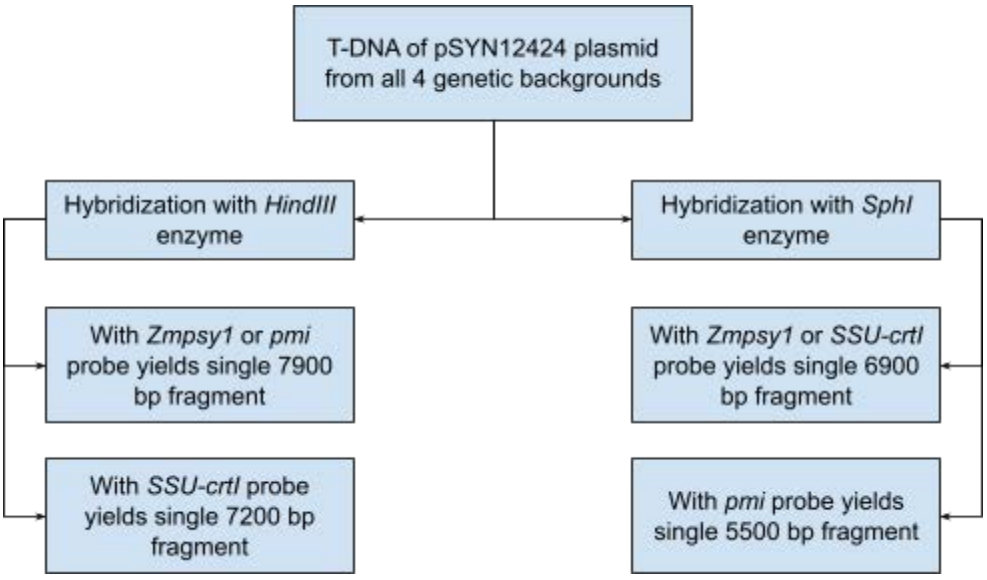

Figure 3.5: *Molecular assessment for the confirmation of genetic changes in GR2E rice*

What does Codex say?

The section 4 point 32 of the Codex document specific for the r-DNA plants (Codex, 2003) that discusses the relevant information needed for a GM plant's modified traits, it requires the information about the gene product i.e. the protein, the level of expression of protein and molecular size and morphology of it as well its functions in the GM plant, along with the phenotypic characteristics through various molecular and biological assays that are possible to confirm for the intended change. Furthermore, assessment for protein in intended sites such as edible portions are done, along with metabolites associated with it are also accounted for.

Guideline for food safety assessment in Bangladesh

The chapter 5 of the Bangladesh document for the guidelines for the GE-plant based food safety assessment (Bangladesh Standards and Testing Institute [BSTI], n.d.), expands on the molecular characterisations of the GM plant, it entails that information about the level of expression of protein and molecular size and morphology of it as well with the phenotypic characteristics through various molecular and biological assays possible for confirmation of the intended change.

Comparison for Harmony of Protocols

Table 3.3 (next page) comparatively illustrates the similarities and differences between the proper assessments to determine the Confirmation of Intended Genetic Changes for the approval of both Anthocyanin Tomatoes and Golden Rice. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003), (Oliva et al., 2020).

Table 3.3: *Comparison of Confirmation of Intended Genetic Change*

Confirmation for intended genetic change Assessment information required	**Anthocyanin Tomatoes (USA)**	**Golden Rice (Philippines)**	**Bangladesh Guidelines**
Assessment for level of protein expression	S.M.	S.M.	S.M.
description of gene function in GM plant	S.M.	S.M.	S.M.
Molecular assessments to determine the protein's size and morphological details	P.M.	P.M.	S.M.
Assessment for Presence/Absence of protein in intended sites, along with metabolites associated	S.M.	S.M.	S.M.

S.M. - Specially Mentioned, P.M. - Partially Mentioned.

3.1.3 Absence of Vector Backbone DNA

Absence of vector backbone DNA means ensuring that the intended r-DNA was fully integrated into the genome of the GM plant and the trait has become more accepted into the plant's physiology. It further ensures that no unintentional trait could be introduced from the vector organism e.g. disease from the *Agrobacterium* used as vector organism (Ladics et al., 2015).

For anthocyanin tomatoes:

According to the food safety assessment report released by the FDA (U.S. Food and Drug Administration [FDA], 2023), NPS had analysed "high throughput sequencing data" from one of the GM tomatoes with the *Del/Ros1-N* trait to investigate the presence of any sequences from the vector backbone that may have gotten integrated into the fruit's genome. NPS concluded afterwards that no vector backbone was found.

For golden rice:

According to the Molecular Characterization and Safety Assessment release by IRRI, no hybridizing fragments were detected when the backbone probes were tested against various samples of GR2E genomic DNA digested by *AscI* & *XmaI*, thus confirming the absence of plasmid backbone sequences. Multiple southern hybridization analyses have clearly demonstrated the insertion of the T-DNA into a single site and the absence of sequences derived from the plasmid backbone. IRRI concluded afterwards that the plasmid vector backbone could not be found.

What does Codex say?

Along not having specific points in regards to assessing for the presence of plasmid vector backbone integration, similar protocols would apply as assessing for formation of any new ORF (refer to section 3.1.5) and for presence of unintended proteins in the GM crop (refer to section 3.1.6) (Codex, 2003)

Guidelines for food safety assessment in Bangladesh

For the guidelines followed by Bangladesh (Bangladesh Standards and Testing Institute [BSTI], n.d.), chapter 4 entails the description of possible genetic changes, hinting for possible changes that can happen from plasmid insertion especially the risks that should assessed, such as, assessment for virulence i.e. safe-use history of the vector plasmid as well as source of plasmid and the isolation and purification process that was involved with sourcing the plasmid for being a vector. Similar information is also taken into account for the *Agrobacterium* or other methods used for insertion of the plasmid into the crop (Bangladesh Standards and Testing Institute [BSTI], n.d.).

Comparison for Harmony of Protocols

Table 3.4 comparatively illustrates the similarities and differences between the assessments to ensure the Absence of Vector Backbone genetic material for the approval of both Anthocyanin Tomatoes and Golden Rice. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003), (Oliva et al., 2020).

Table 3.4: *Comparison of Assessment practices for Absence of Vector Backbone DNA*

Assessment for Absence of Vector Backbone related information required	**Anthocyanin Tomatoes (USA)**	**Golden Rice (Philippines)**	**Bangladesh Guidelines**
Assessment for Native/functional gene cassette disrupted due to plasmid insertion	P.M.	P.M.	P.M.
Confirmation for plasmid safe use history	S.M.	S.M.	S.M.
Confirmation of Vector organism (if any) safe use history	S.M.	S.M.	S.M.
Assessment for Presence/Absence of unintended changes, e.g. proteins that cause harm to crop performance	S.M.	S.M.	S.M.

S.M. - Specially Mentioned, P.M. - Partially Mentioned.

3.1.4 Inheritance Stability of the Desired Trait

This is a necessity to be confirmed when approving the usage of an GM food crop in agricultural use, to ensure that the intended trait is inheritable and so all seeds of the crop will provide a plant that can pertain the same traits as its parent plant i.e. the GM plant (Butelli et al., 2008), (Ladics et al., 2015).

For anthocyanin tomatoes:

In the food safety assessment from FDA (U.S. Food and Drug Administration [FDA], 2023), it was mentioned that NPS had done PCR sequence comparisons of DNA samples from T1 with T6 MicroToms plants and F9 with T6 MicroToms plants to confirm stability of trait inheritance i.e. the stability of the *Del/Ros1-N* trait's gene across generations and genetic backgrounds of these modified fruits. It was concluded by NPS that the inheritance pattern observed followed an "Mendelian segregation fashion" in all tomato genetic backgrounds. "The purple tomato trait has been introgressed into other tomato varieties including Ailsa Craig, Lucinda, VF36, and Ohio

8423 (an industrial processing tomato variety for tomato juice production), which confirmed the stability of the phenotype." (U.S. Food and Drug Administration [FDA], 2023). Figure 3.6 outlines the workflow that determined the inheritance stability in anthocyanin tomatoes.

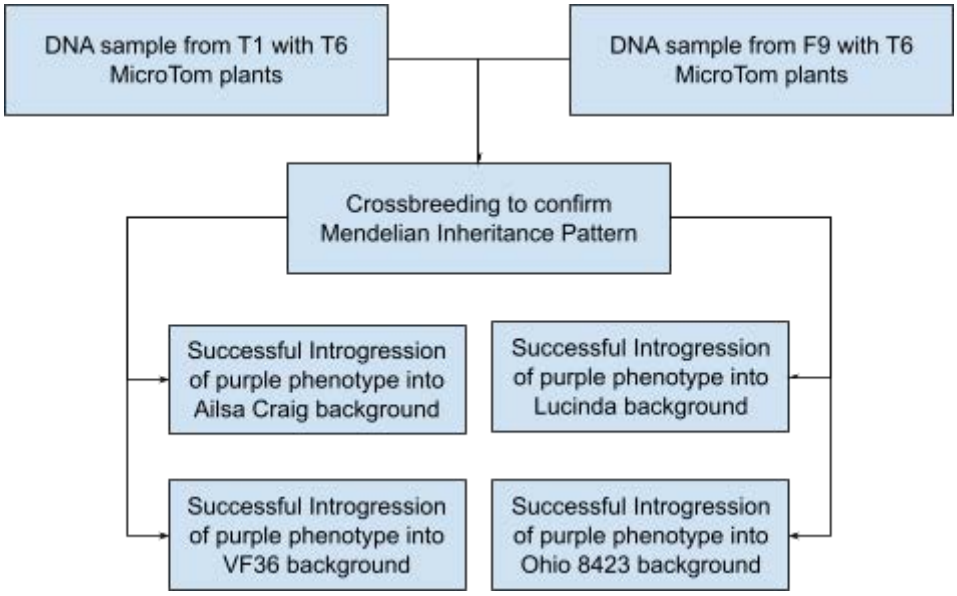

Figure 3.6: *Determination of Inheritance Stability in Anthocyanin Tomatoes*

For golden rice:

Once the initial transformation of GR2E was completed, the resulting transgenic plants were backcrossed with the following non-transformed parent rice varieties: PSB Rc82, BRRI dhan 29 and IR64. The transgenic plants were then self-pollinated and successive generations of offspring were tested for mendelian inheritance patterns and assessed for the stability of the transgenes. Southern blot analysis of genomic DNA samples was performed on samples taken from the (T_n) generation of GR2E in the Kaybonnet background and the following backcrossed generations for each parent; BC_3F_5 (Backcross Generation 3, Filial Generation 5) for BRRI *dhan* 29, BC_4F_3 for IR64 and BC_5F_3 for PSBRc82. Digestions with *HindIII*, *SphI* and a combination of *AscI* & *XmaI* were separated by gel electrophoresis and the blots probed with probes specific for *Zmpsy1*, *SSU-crtI* and *pmi* genes. Single hybridizing fragments of ~7900 bp, ~6900 bp or 8747 bp were detected using the *Zmpsy1*, *SSU-crtI* and *pmi* probes respectively. Furthermore, concentrations of total carotenoids were determined in grain samples collected from GR2E plants in the Kaybonnet germplasm, BC_3F_5 generations in PSBRc82 and IR64 backgrounds and the BC_4F_3 & BC_5F_3 generations in PSBRc82, IR64 and BRRI *dhan* 29 germplasm backgrounds. Carotenoid

accumulation in the endosperm was positively correlated with the presence of the T-DNA insert, thus confirming the stability of the phenotype (Oliva et al., 2020). Figure 3.7 outlines the workflow that determined the inheritance stability of GR2E.

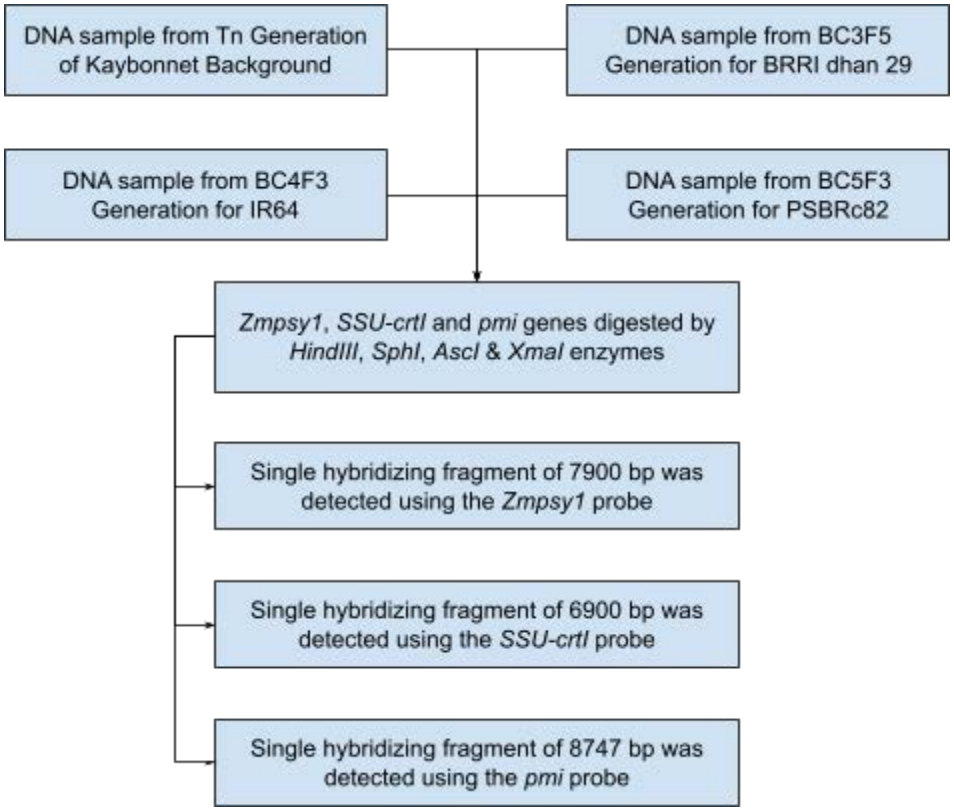

Figure 3.7: *Determination of Inheritance Stability in GR2E Rice*

What does Codex say?

According to the Codex of r-DNA plant food safety assessment (Codex, 2003); the section 4 for general considerations, in the genetic modifications portion of the literature, the point 33(C) describes that information should be provided about the DNA insert in the context for demonstrating if the intended effect of modification/s had been achieved or not, as well as if the intended traits are expressed and inherited in a way that is stable through several generations in a consistent manner that adhere to the laws of inheritance. The document also emphasizes it might be necessary to assess the inheritance of the DNA insert itself or the expression of the corresponding RNA if the phenotypic expression cannot be measured directly i.e. the introduced phenotype does not make the plant visually different from its native/commercial counterpart (Codex, 2003)

Guideline for food safety assessment in Bangladesh

Chapter 5 about molecular characterisation of GM plants from the guidelines used by Bangladesh for GM-based food safety assessment (Bangladesh Standards and Testing Institute [BSTI], n.d.) has detailed listing of the particular informations necessary for assessing the inheritability of the GM traits of a plant; ranging from molecular assay of DNA (e.g. southern blot assay, PCR) to biological assays for phenotypic assessment (e.g. breeding and inheritance of traits in fruits) to ensure that the GM food crop carries stable genetic traits over multiple generations.

Comparison for Harmony of Protocols

Table 3.5 comparatively illustrates the similarities and differences between the relevant assessments to ensure stable inheritance of the transformation in both Anthocyanin Tomatoes and Golden Rice. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003), (Oliva et al., 2020).

Table 3.5: *Comparison of Inheritance Stability*

Inheritance Stability Assessment related information required	**Anthocyanin Tomatoes (USA)**	**Golden Rice (Philippines)**	**Bangladesh Guidelines**
Assessment for Molecular presence of trait's gene	S.M.	S.M.	S.M.
Assessment for trait's protein presence	S.M.	S.M.	S.M.
Assessment to observe the trait being carried on multiple generations	S.M.	S.M.	S.M.

S.M. - Specially Mentioned.

3.1.5 Open Reading Frame Analysis

Open Read Frame (ORF) for a gene refers to regions in the genome which codes for proteins and it includes the regulatory genes involved in the expression of the said trait/protein of the organism. For the safety assessment of any GMO, an assessment of the GMO's ORF is necessary to gain assurance that the genetic cassette of the gene is conserved i.e. the gene is not altered beyond the predicted or intended change/s. Furthermore, it is also important that the modifications done does not affect the gene expressions that are native to the plant and are not intended to be changed in the GM process (Ladics et al., 2015).

For anthocyanin tomatoes:

In the FDA document (U.S. Food and Drug Administration [FDA], 2023), NPS had said to have used bioinformatic analyses to assess if any ORFs were generated/disrupted as a result of the DNA insertion and if so whether the supposed protein translation of any new ORFs raise concern for toxicity or allergenicity that would be relevant for human foods. NPS searched the genomic DNA sequences flanking the insertion in *Del/Ros1-N* tomatoes and found no known ORFs were interrupted by the presence of the *Del/Ros1-N* insertion. NPS also assessed the junction sequence for possible new peptides of equal or greater than 30 amino acids created at the insertion site of the T-DNA in *Del/Ros1-N* tomatoes. One such peptide was identified and analyzed, however this peptide did not have any homogeny to any known allergen or protein and there was no evidence this sequence is transcribed in tomato. So NPS concluded that these results for ORF analysis did not raise food safety concerns (U.S. Food and Drug Administration [FDA], 2023).

For golden rice:

In order to determine whether the possibility of creating new ORFs as a consequence of the T-DNA insertion in GR2E had come to pass, IRRI performed a reading frame analysis to look for potential start-to-stop ORFs that spanned either the 5′ or 3′ junctional regions. After examining each of the three possible reading frames in both orientations for potential ORFs capable of encoding sequences of 30 or more amino acids, 2 ORFs were identified; one in the reverse orientation that spanned the 5′ T-DNA insert-genomic DNA border (ORF-1, 207 bp, 68 amino acids) and one in the forward orientation that spanned the 3′ T-DNA insert-genomic DNA border (ORF-2, 240 bp, 79 amino acids). After comparing the amino acid sequences of the

potential ORFs against a toxin compared to a peer-reviewed database of 2129 known toxic and putative allergens and celiac protein sequences residing in the Food Allergy Research And Resource Program (FARRP) dataset version 19 at the University of Nebraska using the FASTA36 alignment tool using an E-score criterion of ($1 \text{ X } 10^{-5}$), the search resulted in no significant hits returned, leading IRRI to conclude that the ORFs raised no food safety concerns (Oliva et al., 2020).

What does Codex say?

In accordance with the Codex of r-DNA plant food safety assessment (Codex, 2003); section 4 general considerations, in the genetic modifications portion of the literature, the point 31(D) describes that the information should be provided on the DNA insertions into the plant genome. And it should include, from amongst numerous points, the identification of any ORFs within the inserted gene or created by insertion with the contiguous plant genomic DNA including those that could form fusion proteins (Codex, 2003).

Guideline for food safety assessment in Bangladesh

The guidelines for Bangladesh's food safety assessment for GE-based foods unfortunately lack the literature necessary for dictating the assessments needed to assess ORF nature in GM food crops. (Bangladesh Standards and Testing Institute [BSTI], n.d.).

Comparison for Harmony of Protocols

Table 3.6 (next page) comparatively illustrates the similarities and differences between the assessments to ensure the Absence of Vector Backbone genetic material for the approval of both Anthocyanin Tomatoes and Golden Rice. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003), (Oliva et al., 2020).

Table 3.6: *Comparison of Open Read Frame Analysis Assessment*

ORF Assessment related information required	**Anthocyanin Tomatoes (USA)**	**Golden Rice (Philippines)**	**Bangladesh Guidelines**
Assessment for Number of ORFs within the inserted gene	S.M.	S.M.	N.M
Assessment for Number ORFs created due to insertion of gene	S.M.	S.M.	N.M.
Assessment for Native/functional ORF/s disrupted due to insertion	S.M.	S.M.	N.M.

S.M. - Specially Mentioned, N.M. - Not Mentioned.

3.1.6 Assessment of Potential Toxicity

Toxins are undesirable elements that cause an adverse reaction once a sufficient dose is administered to the recipient. So assessing the potential toxicity of the newly expressed proteins in GM food is a critical component of the overall safety assessment. Possible toxins are identified through assessing the similarity of amino acid sequences to known toxins in a peer reviewed toxin database.

For anthocyanin tomatoes:

In the case of the FDA document where NPS did the assessments (U.S. Food and Drug Administration [FDA], 2023), they assessed for the presence of the transcription factors and selective markers present in the end-result *Del/Ros1-N* tomato fruits and whether or not those protein presence could cause any harm upon consumption using Orbitrap Liquid Chromatography-Mass Spectrometry (LC-MS). This was used to measure the concentration of Delila and Rosea1 proteins in the juice made from *Del/Ros1-N* tomatoes grown in greenhouses. NPS stated neither Delila nor Rosea1 proteins were detected in the juice from *Del/Ros1-N* tomatoes. Considering the similar processes to prepare samples for measuring proteins in tomato

fruit and tomato juice, NPS stated Delila and Rosea1 proteins would similarly be below detection levels in the whole fruit. NPS noted that the limit of detection of mass spectrometry is calculated as less than 0.2 ng Rosea1 protein and less than 0.5 ng of Delila protein per milliliter of juice. Since the assessments were done on commercial style juiced tomatoes with no addition of heat, the heat stability assessments were not conducted by NPS so the protein heat stability of Delila and Rosea1 cannot be confirmed (U.S. Food and Drug Administration [FDA], 2023).

NPS used bioinformatic analysis to assess both Delila and Rosea1 genes for sequence similarity to known toxins. NPS found no sequence similarity to known toxins within the T3DB database. NPS explained that the transcription factors Delila and Rosea1 in *Del/Ros1-N* tomatoes are functionally and structurally equivalent to transcription factors that control anthocyanin biosynthesis in commonly consumed fruits and vegetables with a history of safe use.

For golden rice:

To search for similarity to known or potential toxins, the amino acid sequences of *Zmpsy1* and *crtI* were queried against a toxin database using the FASTA36 algorithm. The *Zmpsy1* query sequence did not return any entries with an E-score less than 1×10^{-5}. Therefore, there are no sequence homology alerts for potential toxicity of the *Zmpsy1* protein. However, the *crtI* query sequence returned two protein accessions from the toxin database with an E-score less than 1×10^{-5}. The two sequence alignments were to the conserved N-terminal FAD-binding regions of L-amino acid oxidase (LAAO) enzymes from two species of venomous snakes: *Bungarus multicinctus* and *Bungarus fasciatus*.

The potential for acute toxicity resulting from oral exposure to *crtI* was investigated in mice. Several groups of 5 male and female mice were orally dosed by a gavage with a mixture of bovine serum albumin and purified microbial-expressed *crtI*, at a volume of 13.45 ml/kg BW, administered in two separate doses approximately 4 hours apart on the first day. The test continued for 15 days and all animals survived until the scheduled end of the study period. No clinical signs of toxicity were observed during the test period, nor were any gross lesions found in the mice at necropsy.

What does the Codex say?

When creating recombinant-DNA plants, there is the potential for the modification to express toxic non-nucleic substances in the end product. These points of concern are addressed in Points 34 to 40 of Section-4 of the Codex Alimentarius Guideline for the Safety Assessment of Food Derived from Recombinant-DNA Plants (CAC/GL 45-2003). The safety assessment should take into account the chemical nature and function of these substances and identify the concentration of each in the edible parts of the recombinant-DNA plant, considering the probable effects on various population sub-groups. The decision made regarding its potential toxicity depends on the "Weight of Evidence".

Guideline for food safety assessment in Bangladesh

The Assessment of Potential Toxicity is covered in section 6.1 of Bangladesh's Guide for Safety Assessment of Foods Derived from GE Plants (Bangladesh Standards and Testing Institute [BSTI], n.d.). It lists in detail the necessary information required to confirm that proteins present in the GM plant will be non-toxic to humans and other mammals, and does not represent a hazard under any realistic exposure scenario, including long-term exposure.

Comparison for Harmony of Protocols

Table 3.6 comparatively illustrates the similarities and differences between the relevant assessments to ensure the absence of potential toxins in both anthocyanin tomatoes and GR2E. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with the Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003).

Table 3.7: *Comparison of Toxicity Assessment*

Required information for Toxicity Assessment	**Anthocyanin tomatoes (USA)**	**Golden rice (Philippines)**	**Bangladesh Guidelines**
Pepsin Digestibility	S.M.	S.M.	S.M.
Sequence Similarity to known Toxins	S.M.	S.M.	S.M.
Heat Stability	N.M.	S.M.	S.M.

S.M. - Specially Mentioned. N.M. - Never Mentioned

3.1.7 Assessment of Potential Allergenicity

Unlike toxins, allergies are specific for both the allergen and the person. Which is why no single criterion is sufficiently predictive of potential allergenicity. Therefore, possible allergens are identified through a "weight-of-evidence" based approach. The assessment considers the similarity of amino acid sequences to known allergens, the resistance to pepsin through digestibility under standardized *in vitro* conditions and thermal stability at various temperatures.

For anthocyanin tomatoes:

NPS used bioinformatic analysis to assess both Delila and Rosea1 genes for sequence similarity to known allergens. NPS found no such sequence similarities in either the ADFS database or AllergenOnline database. No identity matches of over 35% identity over 80 amino acids were found. Additionally, there were no sequences of 8 or more contiguous, identical amino acid matches for either Delila or Rosea1 when compared to the sequences of known allergenic proteins.

NPS investigated the stability of Delila and Rosea1 proteins to digestive enzymes by exposure to simulate gastric fluid (SGF) containing pepsin. NPS found Delila proteins are digested by pepsin within 30 seconds while Rosea1 proteins are digested in just under 10 minutes (U.S. Food and Drug Administration [FDA], 2023).

For golden rice:

Potential identities between the *Zmpsy1* and *crtI* query sequence and proteins in the allergen database were evaluated with the FASTA35 sequence alignment tool using the default parameters. A criteria of > 35% identity over any segment of 80 or more amino acids as an indication of possible cross-reactivity to allergens was adopted by the Codex as the primary sequence search criteria for use in flagging proteins that might be of some concern of cross-reactivity for genetically modified plants. No identity matches of > 35% over 80 residues were observed. Also, there were no instances of 8 contiguous identical amino acid matches observed for either *Zmpsy1* or *crtI* when compared with the sequences of known allergenic proteins.

As rapid gastric and intestinal digestion is known to be correlated to the allergenic potential of proteins, the *in vitro* pepsin resistance of native *Zmpsy1* and *crtI* proteins were investigated by exposure to SGF containing pepsin for 30 seconds. No intact proteins were found as assessed by either SDS-PAGE or western immunoblot analysis. Faint, low molecular mass degradation products were visible by Coomassie staining in samples removed at up to two minutes of digestion, but not later.

The thermal stability of *Zmpsy1* was evaluated by measuring enzymatic activity; the conversion of geranylgeranyl diphosphate (GGPP) into 15-cis-phytoene. Enzyme activity was irreversibly destroyed upon heat treatment, with 50% loss of activity following pre-incubation at 42°C for 15 minutes and complete loss of activity at 50°C for 15 minutes. For *crtI*, thermal stability was evaluated by measuring enzymatic activity using a spectrophotometric assay to monitor the conversion of liposome-incorporated 15-cis-phytoene to all-trans-lycopene. Enzyme activity was irreversibly destroyed upon heat treatment, with 50% loss at 51°C for 15 minutes and complete loss of activity following pre-incubation at 55°C for 15 minutes (Oliva et al., 2020).

What does the Codex say?

The issue of allergenicity is addressed in points 41 to 43 of Section-4 of the Codex Alimentarius Guideline for the Safety Assessment of Food Derived from Recombinant-DNA Plants (CAC/GL 45-2003). The protein(s) resulting from the inserted gene, if present in the food, should be assessed for potential allergenicity via an integrated, stepwise, case-by-case approach. The decision made regarding its potential allergenicity depends on the “Weight of Evidence”.

Guideline for food safety assessment in Bangladesh

The Assessment of Potential Allergenicity is covered in section 6.2 of Bangladesh’s Guide for Safety Assessment of Foods Derived from GE Plants (Bangladesh Standards and Testing Institute [BSTI], n.d.). It provides specific information on how to assess the proteins expressed in the GM plant for similarity to any known allergens.

Comparison for Harmony of Protocols

Table 3.7 comparatively illustrates the similarities and differences between the relevant assessments to ensure the absence of potential allergens in both anthocyanin tomatoes and GR2E. Additionally, it highlights the practices necessary for Bangladesh to follow, all in accordance with the Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003).

Table 3.8: *Comparison of Allergenicity Assessment*

Required information for Allergenicity Assessment	**Anthocyanin tomatoes (USA)**	**Golden rice (Philippines)**	**Bangladesh Guidelines**
Pepsin Digestibility	S.M.	S.M.	S.M.
Sequence Similarity to known Allergens	S.M.	S.M.	S.M.
Source of the Introduced Gene	S.M.	S.M.	S.M.

S.M. - Specially Mentioned.

3.1.8 Compositional Analysis

Compositional analysis is necessary to quantify the levels of key nutrients and antinutrients present in the edible portions of the plant, in context of both human and animal nutrition. This includes both the grains and any parts that may be used as feed for livestock. The compounds chosen for testing should be those recognized as key nutrients and antinutrients for the plant species, as per recommendation by OECD consensus documents. Comparative assessment should be performed between the GM plant and an appropriate, unmodified counterpart to assess the range of variation from their conventional counterparts.

For anthocyanin tomatoes:

In order to assess the possible presence of selectable marker NPII, NPS used Orbitrap LC-MS to measure the concentration of NPTII protein in the juice made from *Del/Ros1-N* tomatoes. NPS stated NPTII protein was not detected in the juice. NPS noted that the limit of detection of

LC-MS analysis is calculated as less than 0.2 ng NPTII protein per milliliter of juice. Moreover, NPTII is authorized for use as a food additive in tomato (see 21 CFR 173.170; NPTII is also referred to as aminoglycoside 3′ phosphotransferase II in FDA's regulations) (U.S. Food and Drug Administration [FDA], 2023).

NPS performed an additional analysis to assess or ensure the absence of unintended changes to the levels of components relevant to safety or nutrition. Five *Del/Ros1-N* MoneyMaker Tomatoes and five Control plants were grown in a greenhouse in alternating rows. Fruit collected from a single plant was pooled and treated as one replicate. Compositional Analysis was performed based on the principles outlined in the OECD Consensus Document for tomato composition. The report described the levels of the following components in the *Del/Ros1-N* tomatoes and control plants:

1) Proximates
2) Amino acids
3) Fatty acids
4) Vitamins
5) Minerals

NPS states that the levels of the components in *Del/Ros1-N* tomato and the Control are similar or possess minor differences; the levels for total folate, lycopene, β-carotene and α-tomatine are 25% higher in the *Del/Ros1-N* tomato compared to the control. NPS notes, however, that the levels of all tested components in the *Del/Ros1-N* tomato and the control are within the ranges reported in the dossier.

After performing a comparative analysis of the tomato juice between tomato *Del/Ros1-N* tomato (in Ohio 8423 genetic background) and non-transformed wild-type Ohio 8423 control tomato, NPS concluded that the juice from the *Del/Ros1-N* tomato is similar to that of the control, and the nutritional composition of the juice is similar to that of whole tomato fruit. NPS finished by stating that *Del/Ros1-N* tomato is as safe and as nutritious as other commercial tomatoes.

For golden rice:

In order to determine any significant changes in the composition of key nutrients and antinutrients in GR2E rice as compared to its traditional counterpart, samples of paddy rice, straw and bran were taken from both GR2E (in PSBRc82 background) and nontransgenic, near-isogenic, control rice (PSBRc82) cultivated over the 2015 wet growing season and 2016 dry growing season in the Philippines.

Four sites were used to grow the rice: PhilRice-Batac Station, Robert S. Zeigler Experimental Station, PhilRice Central Experimental Station and PhilRice Isabela Station. Planting and plot maintenance practices, such as pest and disease control measures, were in accordance with local agronomic practices for rice production. Three blocks (replicates) of each entry, both the GR2E and traditional lines, were established at each test site in a randomized complete block design. Each entry was planted in 10-row plots of 5 meters in length for a total plot area of 10 m^2. There were 25 plants per row, totalling 250 plants per entry per plot. Harvested grain and straw samples were frozen and shipped to EPL Bio-Analytical Services, where in accordance with guidance contained within the OECD consensus document on compositional considerations for new rice varieties, the compositional parameters for nutrients and antinutrients were analyzed in the paddy rice, straw and bran of the GR2E and Control Rice.

Following the compositional analysis of GR2E and the control rice samples, EPL Bio-Analytical Services determined that there were no statistically significant differences in the GR2E stockline as compared to the control rice sample for the following parameters:

1) Proximate, Fiber and Mineral Composition
2) Amino Acids
3) Fatty Acids
4) Vitamins
5) Antinutrients: Phytic Acid & Trypsin Inhibitor

The only notable exception were the carotenoids, where the values present within the control PSBRc82 rice were below the Level of Qualification, as compared to the GR2E rice.

Within the 62 compositional parameters quantified in the derived samples, there were no meaningful differences between GR2E and the control PSBRc82 rice, except for the intended production of provitamin A carotenoids. With that sole exception, the compositional parameters measured in samples of GR2E rice were within or similar to ranges reported for conventional rice varieties with a history of safe consumption. Overall, no consistent patterns have emerged to suggest that biologically meaningful changes in composition or nutritive value of the grain, straw or bran had occurred as an unexpected and unintended consequence of the genetic modification. Other than the intended production of β-carotene and related provitamin A carotenoids in the endosperm, GR2E rice was found to be compositionally equivalent to conventional rice.

What does the Codex say?

In order to establish that nutritionally significant substances have not been altered in a manner that would have an adverse impact on human health, the concentrations of key components of the recombinant-DNA plant should be compared to an equivalent analysis of their conventional counterpart, grown and harvested under the same conditions. The statistical significance of any differences may then be assessed in the context of the range of natural variations for that parameter to determine its biological significance (Codex, 2003).

Guideline for food safety assessment in Bangladesh

Compositional Analysis is covered in section 6.3 of Bangladesh's Guide for Safety Assessment of Foods Derived from GE Plants (Bangladesh Standards and Testing Institute [BSTI], n.d.). It lists how to assess the variation between the GE plant and an appropriate conventional counterpart.

Comparison for Harmony of Protocols

Table 3.8 comparatively illustrates the similarities and differences between the relevant assessments to ensure proper Compositional Analysis for both anthocyanin tomatoes and GR2E. Additionally, it highlights the practices necessary for Bangladesh to follow, in accordance with

the Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003).

Table 3.9: *Comparison of Compositional Analysis*

Required information for Compositional Analysis	**Anthocyanin tomatoes (USA)**	**Golden rice (Philippines)**	**Bangladesh Guidelines**
Concentration of nutrient and antinutrient values in the food	S.M.	S.M.	S.M.
Assessment of meaningful changes in the nutritional composition	S.M.	S.M.	S.M.

S.M. - Specially Mentioned.

3.1.9 Intended Nutritional Modification: Dietary Exposure

The modification of the nutritional quality of food merits additional nutritional assessment to assess the consequences of the altered nutrient intake changes and what the effects of its introduction into the food supply may be. It is possible to estimate the probable average and maximum intake of the altered nutrient profile by analysing historical food consumption patterns and their nutritional implications. Modeling the estimate after the highest likely consumption rate helps ensure all potentially undesirable effects will be detected. When creating these estimates, the physiological characteristics and metabolic requirements of specific population subgroups, ie; infants, pregnant women, elderly, etc. must be considered.

For anthocyanin tomatoes:

The intended uses of tomato as food are clearly stated. Thus, the FDA approved that unprocessed anthocyanin tomato may be used as an ingredient in salads, and when processed, that it may be used to create tomato juice, puree and sauce (U.S. Food and Drug Administration [FDA], 2023). According to the published report, the intended use of *Del/Ros1-N* Tomatoes as human food

show no notable variance to their unmodified counterparts outside the intended changes; upregulated anthocyanin production.

The intended goal for the development of Anthocyanin Tomatoes was to genetically upregulate anthocyanin expression in tomatoes. In *Del/Ros1-N* Tomatoes, this is characterized by the increased production of the anthocyanin species delphinidin 3-O-(coumaroyl) rutinoside-5-O-glucoside and petunidin 3-O-(coumaroyl) rutinoside-5-O-glucoside, with additional upregulation in some other anthocyanin species. The safety assessments of anthocyanin tomatoes revealed that while anthocyanin concentration in unmodified tomatoes were undetectable, the concentration was much higher in the modified tomatoes. Hemizygous *Del/Ros1-N* MicroTom Tomatoes averaged at 2.83 ± 0.46 mg of Anthocyanin per gram while the *Del/Ros1-N* MoneyMaker Tomatoes averaged at 0.4 mg.

NPS, utilizing the 2009-2010 cycle of National Health and Nutrition Examination Survey (NHANES) data, estimated the concentration of consumed Anthocyanins from *Del/Ros1-N* Tomatoes over a 2 day period, assuming complete replacement of unmodified tomatoes in the diet. The mean consumption stood at 100 mg/day, while the 90th percentile stood at 225 mg/day. This is equivalent to regular consumption of unmodified high anthocyanin foods, ie; blueberries, and falls within safe limits.

For golden rice:

As the intended use for GR2E rice is as food, immunoblot analysis and quantitative ELISA were used to both assess the tissue specificity and concentration of *Zmpsy1*, *crtI* and *pmi* within tissue samples collected from GR2E rice. This information was used in order to determine the results of exposure on humans and animals through their diet.

Three replicated samples of three stages of grain (milky, dough, mature) and straw were collected from GR2E rice grown at four locations in the Philippines during two growing seasons across 2015 and 2016. The expression of *Zmpsy1* and *crtI* genes in sample tissues were consistent with the activity of rice endosperm-specific GluA-2 promoter, meaning that they were detected in all three stages of grain but not in samples of bran, hull or straw tissue. In

comparison, the expression of the *pmi* gene was driven by the constitutive maize polyubiquitin promoter and it was detected in all rice tissues tested.

The overall highest concentrations of *Zmpsy1* and *crtI* were measured in samples of dough-stage grain, ranging between ca. 308–359 ng/g fresh weight tissue (FWT) and between ca. 54–68 ng/g FWT respectively. The highest concentrations of *Zmpsy1* and *crtI* measured in samples of mature grain were ca. 245 ng/g FWT and 30 ng/g FWT, respectively. Concentrations of *pmi* protein were significantly higher than either *Zmpsy1* or *crtI* in samples from all grain growth stages, and were highest in dough-stage grain, averaging ca. 2015 ng/g FWT. The mean *pmi* concentration in mature GR2E rice grain samples was ca. 1282 ng/g FWT. It was also present in straw samples at concentrations ranging between 320–796 ng/g FWT, with an average concentration of ca. 482 ng/g FWT.

Two approaches were used to derive the possible daily rice consumption of Golden Rice. The first approach used the historic rice utilization data for the highest rice-consuming countries in Asia, compared to the United States, obtained from the USDA Production Supply and Distribution database and converted to per capita utilization estimates using the FAOSTAT population database. Projected utilization values for the same countries were obtained from the International Rice Outlook: International RiceBase Projections. Using the highest projected per capita rice utilization in Cambodia of 253 kg/yr and an estimated average adult body weight of 57.7 kg in Asia, the maximum daily rice intake was calculated as shown:

$$\text{Daily Rice Intake} = [253\ (\text{kg/yr}) / [365 \times 57.7\ (\text{kg BW})]] \times 1000\ (\text{g/kg}) = 12\ (\text{g/kg BW})$$

The second approach utilized data from the combined database created by the cooperation of the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), the Chronic Individual Food Consumption Database summary statistics (CIFOCOss), currently containing summary statistics of 37 surveys from 26 countries.

Based upon consideration of both approaches, a value of 12.5 g/kg body weight was chosen as the upper limit of mean daily dietary intake of rice, a value which was considered sufficient to

account for consumption by all population subgroups. To derive the estimates of maximum potential daily dietary intake of the *Zmpsy1*, *crtI* and *pmi* proteins expressed in GR2E rice, three assumptions were used:

(1) The mean daily dietary rice consumption is 12.5 g/kg body weight

(2) 100% percent of the dietary rice intake is from GR2E rice

(3) The grain concentrations of *Zmpsy1*, *crtI* and *pmi* used for estimation are the highest values measured

Using these assumptions, the estimated maximum potential daily dietary exposure from GR2E rice to each novel protein is estimated to be ca. 4.5, 0.85, and 30 μg/kg body weight for *Zmpsy1*, *crtI* and *pmi* proteins respectively.

What does the Codex say?

Annex-2 of the Codex Alimentarius states that foods derived from recombinant-DNA plants that have undergone modification to intentionally alter their nutritional quality or functionality should be subjected to additional nutritional assessment to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply (Codex, 2003).

Guideline for food safety assessment in Bangladesh

Intended Nutritional Modification is addressed in section 6.4 of Bangladesh's Guide for Safety Assessment of Foods Derived from GE Plants (Bangladesh Standards and Testing Institute [BSTI], n.d.). It addresses the additional assessments required when the nutrient profile of a recombinant-plant undergoes a qualitative and quantitative change.

Comparison for Harmony of Protocols

Table 3.8 (next page) comparatively illustrates the similarities and differences between the required assessments for Nutritional Modification for both anthocyanin tomatoes and GR2E. Additionally, it highlights the practices necessary for Bangladesh to follow, in accordance with the Codex Alimentarius (Bangladesh Standards and Testing Institute [BSTI], n.d.), (Codex, 2003).

Table 3.10: *Comparison of Nutritional Modification*

Required information for Nutritional Modification	**Anthocyanin tomatoes (USA)**	**Golden rice (Philippines)**	**Bangladesh Guidelines**
Probable daily intake of the altered nutrient profile	S.M.	S.M.	S.M.
Assessment of possible detrimental effects on population sub-groups	S.M.	S.M.	S.M.
Assessment of stability of altered nutrient in context of processing and storage	S.M.	S.M.	S.M.
Determination of bioavailability of altered nutrients	S.M.	S.M.	S.M.

S.M. - Specially Mentioned.

Chapter 4: Discussion and Conclusion of Key Concepts

The comparisons tabulated as well as explained in this thesis shows that the USA, the Philippines and Bangladesh attempt to adhere to the international guidelines from the Codex when creating their respective food safety assessment. It shows that roughly for all aspects the two cases as well as the guideline of Bangladesh follows the Codex very carefully. The case-based comparative analysis focusing on molecular characteristics of GM foods showed how the approved GM foods were adopted in their respective country. This allowed a better understanding on the basic criteria in regulatory practices that get overlapped and so are prioritised for the food safety assessment process. Bangladesh for what this comparative analysis showed, has a guideline that is more detailed when brought for comparison with the specified cases and this is due to a deeper interpretation of the Codex Alimentarius. Therefore the commonalities in practices as well as the already established detailed guideline of Bangladesh, this country can conduct food safety assessments of nutritional GM food with greater details oriented process and with more assurance of safe consumption of such nutritional GM foods.

Furthermore, based on the comparative analysis results of this thesis, it can also be concluded that protocol harmonization in context for adopting the usage of nutritional GM foods is possible for Bangladesh. The following flowchart (figure 4.1) entails a suggested workflow for how Bangladesh may allow the adoption of GM food that have nutritional benefits, based on the existing guidelines as well as some new considerations that adhere to the Codex.

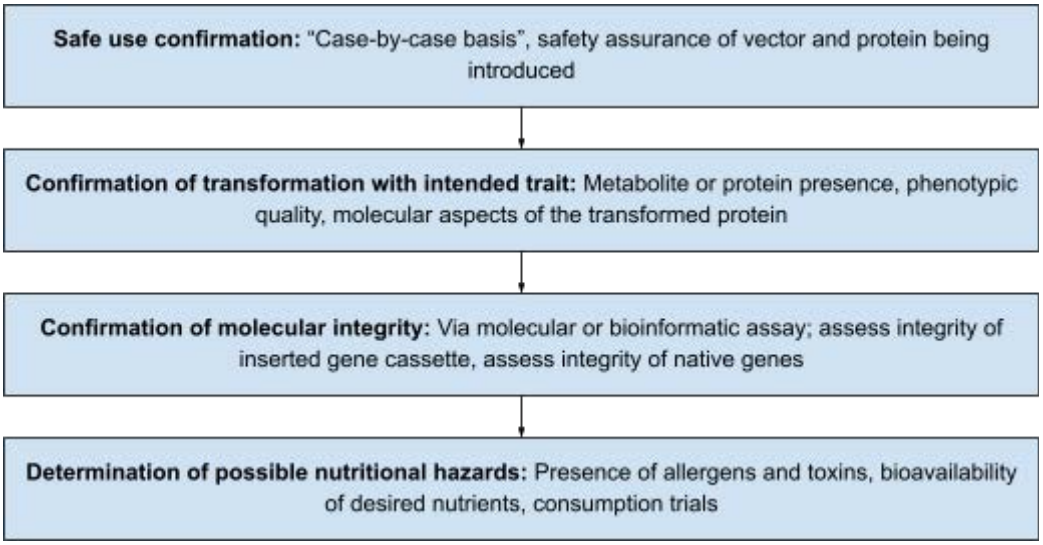

Figure 4.1: *Suggested workflow for food safety assessment that can be followed in Bangladesh for assessing safety of GM Food modified for nutritional benefits*

In this thesis, we strived for a clear regulatory harmonization by reflecting on the approved GM foods in the Philippines and the USA, one a developing country and the other a developed country, by using two different GM foods that served different dietary purposes in their respective countries. However, this approach of using Anthocyanin Tomatoes and Golden Rice approval processes fall short of the finer assessment points that could have been explored if comparing the approval process of the same GM food in the two countries. For example, Golden Rice is approved in both aforementioned countries, so the comparison of this food could have allowed those points to be explored in depth.

However realistically, the US consumes less rice compared to the Philippines, an Asian country that predominantly relies on rice as a main source of carbohydrates. The case for Anthocyanin Tomatoes for the USA was more suited for the purposes of this thesis, as it is a commonly consumed vegetable on a global scale. The cases used with GM tomato and GM rice was useful for the comparative approach attempted in this thesis, as it allowed for the versatility of the same regulation in adapting the “case-by-case” scenarios of GM food approvals to shine through. Thus showcasing in a simpler manner that protocol harmonization is possible in broad aspects.

The USA case highlights how a developed country with a high record of GM food approval manages to maintain regulatory practices to ensure safe usage for humans (Jacobsen et al., 2013), (U.S. Food and Drug Administration [FDA], 2024). Even though the Philippines and Bangladesh are developing countries of similar agro-socio-economic circumstances compared to the USA, it still leaves room for discussion of regulatory overlaps for such developing countries. As mentioned before, Bangladesh has a pending decision on approval of Golden Rice, having similar agro-socio-economic conditions as the Philippines might help for a smoother approval process than that with a country like the USA.

Bangladesh, USA and Philippines all follow and have adapted the regulations of The Codex Alimentarius into their regulatory practices; thus for the purposes of this thesis, this regulatory commonality between the countries allowed for understanding what truly matters for approvals of GM food, which can be vital for their nutritional benefits as we tried to exhibit in this thesis.

For possibilities for further research in this concept, a similar comparative study can be attempted with the same GM foods approval workflow in two countries that consume the food similarly. For example if Golden Rice was approved for consumption in Bangladesh then such a study could be conducted for protocol of Bangladesh and Philippines to compare finer assessment points. For a country like Bangladesh that needs to ensure food security for its growing population, creating ways to allow regulatory harmonization would effectively help with resolving malnourishment, creating a better future for the country as a whole.

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