PhD DISSERTATION PROJECTS

**Tomato and eggplant fruit fortification by gene editing of Glutathione S-transferase (GST) loci**

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This PhD thesis research project is aimed at enhancing the nutritional quality of the fruit in tomato by CRISPR/Cas9-mediated gene editing approaches for removing inhibitory factors of GST gene expression.

**Bio-fortificazione del frutto di pomodoro e melanzana mediante gene editing di loci Glutathione S-trasferasi (GST)**

Questo progetto di tesi di dottorato mira ad incrementare l’espressione di geni GST mediante gene editing con tecnologia CRISPR/Cas9 volta a rimuovere la repressione operata da specifici geni regolatori e incrementare l’accumulo di antiossidanti e la qualità nutrizionale del frutto in pomodoro.

# **1. State-of-the-Art**

Tomato (*S. lycopersicum*) is a worldwide cultivated food crop and its global yield has increased over the past decade. Its fruit contain many health-promoting compounds that have been involved in the prevention of several chronic diseases and dysfunctions (Frusciante et al., 2007. In addition to its economic and nutritional importance, tomato is an important model plant for scientific research on fruit development and quality. Since its first release in 2012, the tomato genome sequence has been widely used as a reference genome for scientific research and biotechnology assisted breeding approaches.

In order to control the overall level of reactive oxygen species (ROS) and prevent cellular damage and lipid peroxidation, the plant metabolic processes deploy a plethora of bioactive compounds with antioxidant activity as defense shield against oxidative stress. The antioxidant system consists of several metabolites and enzymatic proteins such as glutathione S-transferase (GSTs). GSTs are phase II metabolic isozymes that catalyze the conjugation of the tripeptide (γ-Glu-Cys-Gly) glutathione (GSH) to a variety of substrates such as endobiotic and xenobiotic compounds for the detoxification. GSH is a key player in the plant response to the oxidative stress. In fact, GSH biosynthesis is stimulated when the cell faces stress conditions and builds up its defense capability. Also, GSH collaborates with ascorbate and NADPH in the Foyer-Halliwell–Asada cycle (Potters et al., 2002) for H2O2 detoxification preserving cells from damages brought about by exceeding levels of ROS.

In plants, GSTs exist as a multigene superfamily and can be grouped in cytosolic, mitochondrial and microsomal. Based on their sequence plant GSTs are categorized in distinctive classes, that are tau (U), phi (F), theta (T), zeta (Z), lambda (L), dehydroascorbate reductase (DHAR), γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ), tetrachlorohydroquinone dehalogenase (TCHQD), metaxin, Ure2p, hemerythrin (H), iota (I), microsomal prostaglandin E-synthase type 2 (mPGES-2) and glutathionyl hydroquinone reductase (GHR) (Ref.). Phi and tau are the largest plant GST classes.

Within the tomato genome 90 GST genes unevenly distributed across the 13 synthetic chromosomes has been previously identified (Islam et al. 2017). Among others, chromosomes 7 and 9 harbor the highest number of GST genes, 23 and 12, respectively. Moreover, GST genes are grouped in 10 clusters and the two major tau cluster are located on chromosomes 7 and 9.

Previous research carried out in our laboratory allowed us to associate in tomato GST up-regulation with fruit accumulation of phenolic compounds (Di Matteo et al., 2013). However, the use of GST genes to improve the nutritional quality of the fruit remains largely unexplored given the complexity of this gene family with a high degree of redundancy and duplication that limit the specificity of many metabolic engineering technologies and the high homeostatic strength that allows the system to react and debunk or cancel any effect of externally-induced unbalance. To functionally characterize candidate genes and breed tomato for enhanced fruit nutritional quality, genome editing technologies relay on DNA repair mechanisms that occur in cells either by non-homologous end-joining or homology directed repair systems (Steinert et al., 2016). Editing technologies, can efficiently achieve site-directed mutations of target sequences and further removal of foreign DNA by segregation in order to deal with regulatory and public acceptance issues.

# **2. PhD Thesis Objectives and Milestones**

Our aim is to use gene editing technology that has some strengths such as: the possibility of drawing RNA specific guide to target individual loci even within families with many duplications and the possibility of drawing partially non-specific guide RNA in in order to simultaneously target genes of the same subfamily and limit the homeostasis reaction.

Another innovative element of the project proposal is the possibility of pursuing metabolic alterations by editing loci miRNA to remove the inhibition on the target gene.

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Target selection** among cluster 7 and cluster 9 in order to design specific gRNAs (A1.1) and purchase PCR primers for cloning (A1.2).

A2) **Purification of vectors (A2.1) and cloning (A2.2)** of gRNAs into appropriated entry and destination vectors in order to prepare binary vectors for CRISP/Cas9 applications by GoldenBraid 3.0 technology.

A3) **Plant transformation.** Agrobacterium tumefaciens LBA4404 will be transformed with cloned binary vectors and appropriate cell cultures will be set for co-cultivation (A3.1). tomato leaf and hypocotyl explants will be co-cultivated and incubated for callus development. Callus undergo shoot regeneration and further rooting on appropriate media (A3.2). Rooted plants will be propagated in vitro and adapted to in vivo environment in an appropriated plant growth chamber.

A4) **Molecular, eco-physiological and nutritional characterization of mutants**. In order to better understand the effect of the induced knock-out, selected F2 edited plants undergo genomic DNA extraction and PCR target amplification and sequencing for validating the severe mutation. Similarly, predicted off-target mutations will be checked by sequencing. Expression analysis of GSTs sharing high sequence similarity will be performed by TaqMan qPCR. Similarly, the effect on the regulation of genes involved into the response to the antioxidant stress and antioxidant biosynthesis will be analysed by qPCR (A4.1). Plants grow in an appropriate greenhouse will be characterized for leaf photo-assimilation efficiency, response to antioxidant stress, fruit set-up and yield (A4.2). Fruits will be analysed for antioxidant profiling (A4.3).

A5) **Recombination and T-DNA elimination (A4).** In order to eliminate the engineered T-DNA, edited plants will be self-pollinated and F2 segregants will be selected for keeping the edited alleles and missing the T-DNA insertion by using appropriate TaqMan molecular markers.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

***Table 2***Gantt diagram for this PhD thesis project.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Activity Months | | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** |
| A1) | ***Target selection*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Design of gRNAs |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 2) Design and purchasing of PCR primers |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A2) | ***Purification of vectors and cloning*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Purification of vectors |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 2) Cloning |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A3) | ***Plant transformation*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Preparation of *A. tumefaciens* cultures |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 2) Preparation of explants, co-cultivation, callus development and regeneration |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A4) | ***Charachterization*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Molecular characterization |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 2) Ecophysiological characterization |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 3) Nutritional phenotyping |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A5) | ***Ricombination and T-DNA elimination*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A6) | ***Thesis and Paper Preparation*** |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

# **3. Selected References**

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