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TESI DI LAUREA

New biotechnological approaches in Globe Artichoke: PPOs mining and setting up of genetic transformation protocols

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List of abbreviations:

ABE - adenine base editing
AS - Acetosyringone
AtU6 26 - *Arabidopsis thaliana* U6 26
A. thaliana - *Arabidopsis thaliana*
A. tumefaciens - *Agrobacterium tumefaciens*
amiRNAs - Artificial micro RNAs
BAP - benzyl-aminopurine
BBM - Baby boom
C3 - Romanesco C3
Cas - CRISPR-associated protein
CaMV35S - *Cawliflower Mosaic Virus* 35S
CBE – cytidine base editing
C. cardunculus - *Cynara cadunculus*
CBTs - Conventional breeding techniques
Chr - Chromosome
CK - cytokinin
CLV 3 - CLAVATA 3
CRISPR/Cas9 - Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
crRNA - CRISPR RNAs
cTP - Chloroplast transit peptide
CuA - Diccoper center A
CuB - Dicopper center B
DNA - Desoxyribonucleic acid
DSBs - Double strand breaks
E. coli - *Escherichia coli*
ETGM - Established techniques of genetic modification
GFP - Green fluorescent protein
GUS - ß-glucoronidase
HNH - Histidine-asparagine-histidine
HR - Homologous recombination
IPT - Isopentenyl transferase
Indel - Insertion/deletion
Kan - kanamycin
mRNA - messenger RNA
MS - Murashige & Skoog
NAA - 1-naphtaleneacetic acid
NPTII - *neomycin phosphotransferase*
NHEJ - Non-homologous end joining
NBTs - New breeding techniques
NOS - Nopaline synthase
OD - Optical density
ORF – Open reading frame
OTs - Off-target sites
PAM - Protospacer adjacent motif
PDS - Phytoene desaturase
PEG - Polyethylene glycol
PPOs - Polyphenol oxidases
PTGS - Post-transcriptional gene silencing
REC - recognition domain
RIF - Rifampicine
RNAi - RNA interference
RTqPCR - Real time quantitative polymerase chain reaction
RuvC - metal-ion-dependent nuclease domain
SAM - Shoot apical meristem
sgRNA - Single guide RNA
SP - Spinoso di Palermo
S. pyogenes - *Streptococcus pyogenes*
STM - Shoot meristem less
T0 - time zero
T15 - 15 min after wounding
T-DNA - Transfer DNA
TALEN - Transcription activator-like effector nucleases
TIM - Timentin
tracrRNA - trans-activating CRISPR RNAs
VS - Violetto di Sicilia
VT - Violetto di Toscana
WUS - Wuschel
X-Gluc - 5-bromo-4-chloro-3-insolyl-ß-D-glucoronide
ZFNs - Zinc finger nucleases

1. INTRODUCTION

1.1 Globe artichoke (*Cynara cardunculus*)

1.1.1 The plant and its genome

Globe Artichoke (*Cynara cardunculus* var. *scolymus* L.) belongs to the *Asteraceae* family, one of the largest Angiosperm families (24,000-30,000 species and 1,600-2,000 genera) distributed worldwide (https://www.asteraceaegenomesize.com/). *Cynara cardunculus* (*C. cardunculus*) includes three botanical taxa: *C. cardunculus var. scolymus* (the globe artichoke) (Figure 1a), var. *altilis* (the cultivated cardoon) (Figure 1b) and var. *sylvestris* (the wild cardoon) (Figure 1c). They are cross-pollinated diploid species with highly heterozygous genetic background. Both *C. cardunculus* cultivated varieties evolved from wild cardoon thanks to anthropogenic selection.

Globe artichoke is consumed and cultivated in the Mediterranean Basin; Italy is the main worldwide producer in terms of quantity and has the largest artichoke biodiversity (Pandino et al., 2011). In fact, globe artichoke domestication took place mostly in Italy, where there are more than 120 varietal types classifiable by harvest time and capitulum traits (shape, dimension, spines, pigment of the outer bracts). These characteristics discriminate four main varietal types: Spinosi, Catanesi, Romaneschi and Violetti (respectively Figure 1d, 1e, 1f, 1g) (Acquadro et al., 2020).

In the past few years, the whole genome sequence of different globe artichoke genotypes has been sequenced, and structurally and functionally annotated, simplifying genetic analyses. The whole globe artichoke genome sequence has been released by a consortium including the **University of Turin** (Unit of Agricultural Genetics), the **University of California** (The Genome Center) and the University of Catania (Di3A) within the framework of the **Compositae Genome Project.** The first globe artichoke genome draft (v.1.0) was assembled by Scaglione and colleagues in 2016 (Figure 1h). The genome was assembled into 13K scaffolds which comprise 725 Mb of genomic sequence with a *de novo* prediction of 26,906 genes. The following year, the **resequencing analyses** of **four globe artichoke genotypes**, representative of the core varietal types, as well as a genotype of the related taxa cultivated cardoon was performed (Acquadro et al., 2017). Recently, based on v.1.0 sequencing data, a new reference genome assembly (v.2.0) was generated (Acquadro et al., 2020) (Figure 1h). This genome improved different scaffolds of the v.1.0 genome sequence and reduced the cumulative size of unplaced scaffolds by increasing the anchored genome sequence from 73% (of v1.0) to 94%. It also led to a new accurate gene prediction (28,632 genes).

1.1.2 Economic evidence of globe artichoke

The origin of domestication of globe artichoke placed in South Italy, later new selected genotypes, were introduced in Sicily, like Violet de Provence, from France, and Romanesco. This importation has led to a market expansion, but also to a negative impact on local germplasm. Analysis of genic markers (AFLP fingerprinting) showed a genetic variation within populations, suggesting the multiclonal composition of varietal types (Portis et al., 2005). These results highlight the importance of on-farm germplasm preservation strategies.

Globe artichoke production, in terms of tons harvested, is leaded by Italy, Egypt and Spain with respectively 24.1%, 20.3% and 12.9% of the worldwide products harvested in 2020 (FAOSTAT, 2020) (Figure 1i). In Italy, the hectares occupied by globe artichoke cultivation are nearly double more than in Egypt and in Spain. Globe artichoke production has been stable over years, with around 1.5 million tons harvested per year, from 2010 to 2020, maintaining Italy as the main producer (FAOSTAT, 2010-2020). The worldwide production values about 1 billion US$ with 3,7 million deriving from the Italian production (FAOSTAT, 2020).

Globe Artichoke is economically important both in food system and as medicinal plant. The edible part of artichoke is the immature inflorescence, the capitulum, comprehensive of external and internal bracts and receptacle (Pandino et al., 2011). The inflorescence represents 30-40% of the entire artichoke fresh weight. About 80-85% of the total biomass of the plant is discarded from the industry and, in a circular economy vision, this amount of discard can be recycled producing food additives and nutraceuticals (de Falco et al., 2015). Another way to value the biomass discarded from the cultivation of globe artichoke could be the production of lignocellulosic biomass for pellets as solid biofuel. Considering that the areas of production are restricted (Sicily, Sardinia and Apulia), the use of this biomass for bioenergy can reduce the costs of transport (Raccuia et al., 2013).

Globe Artichoke is an important source of minerals, fiber and polyphenols for the human diet (Grabowska et al., 2018) and also contains pharmaceutically and nutraceutically active compounds like chlorogenic acid and cynarin (1,3-dicaffeoylquinic acid) (Fratianni F. et al., 2007). In addition, Lutz et al., (2011) proved that the process of cooking increases the content of polyphenolic compounds, therefore the radical scavenging capacity raises, so does the quality.

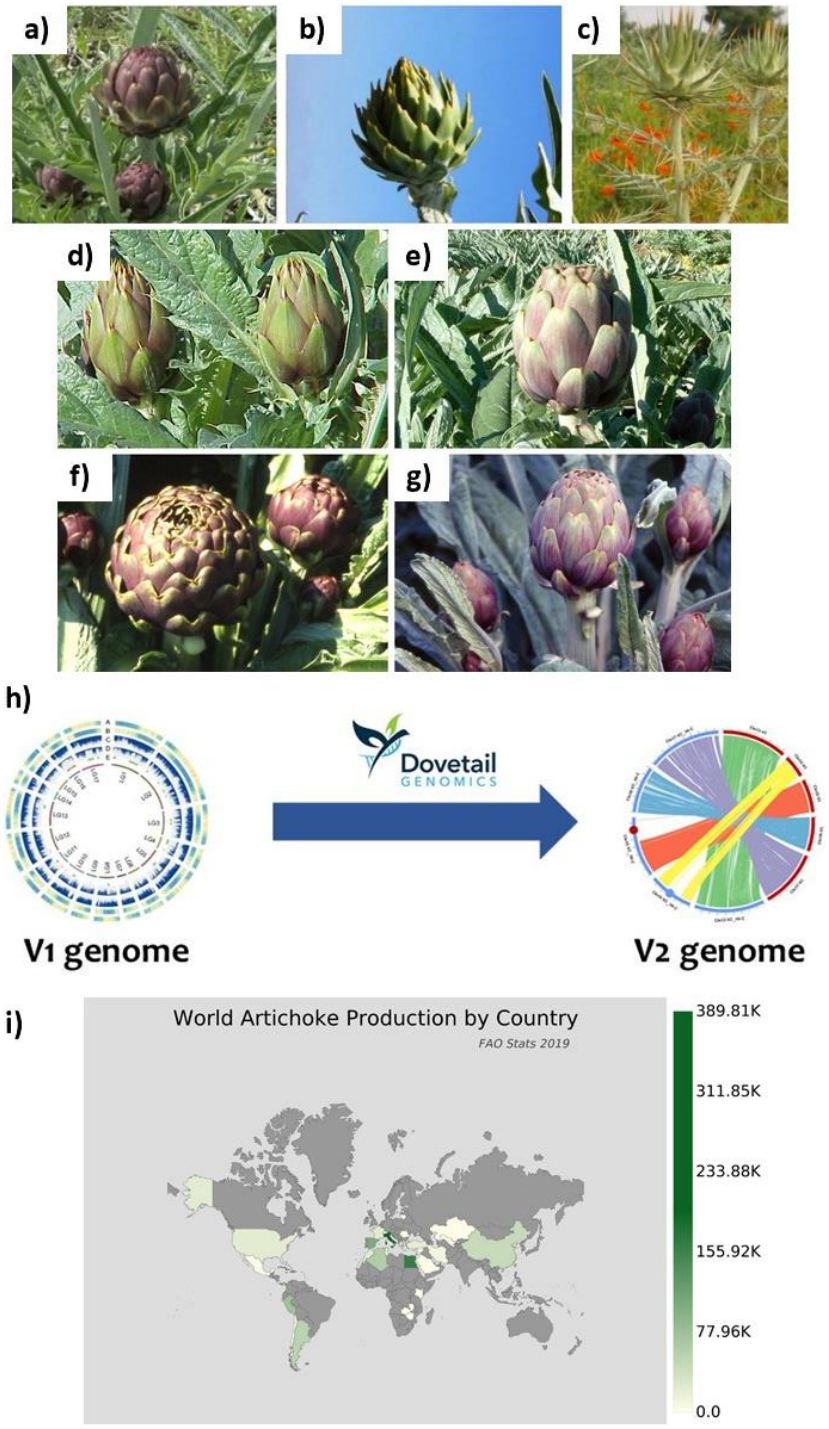


Figure 1. Overview on the globe artichoke plant, its genome and worldwide production. (a-c) Pictures showing *C. cardunculus* botanical taxa: *C. cardunculus* var. *scolymus* (a), var. *altilis* (b) and var. *sylvestris* (c) (http://www.artichokegenome.unito.it/globe-artichoke/). (d-g) Pictures showing the four main varietal types in globe artichoke: Spinosi (d), Catanesi (e), Romaneschi (f), Violetti (g) (http://www.artichokegenome.unito.it/globe-artichoke/). (e) Schematic representation of v1.0 and v.2.0 globe artichoke reference genomes (Acquadro et al., 2020; Scaglione et al., 2016). f) Illustration of the worldwide globe artichoke production, in terms of tons, in 2019 (FAOSTAT, 2019).

1.2 Polyphenol oxidases

After harvesting and during capitulum industrial processing, the activity of polyphenol oxidases (PPOs) in globe artichoke starts leading to an enzymatic reaction which results in browning of tissues (Figure 2a). This enzymatic reaction is particularly associated with senescence and wounding and has negative effects on flavor and nutritional properties, and it also reduces the shelf life of the product (Taranto et al., 2017).

1.2.1 PPOs and their biochemical activity in *planta*

The PPOs are dicopper enzymes that oxidize phenols to correspondent quinones, *ortho*-diquinones, using molecular oxygen (Figure 2b). The generated quinones are highly reactive and they can cross-link or alkylate proteins resulting in brown pigments formation (e.g. melanin) (Figure 2b) observed in damaged plant tissues and in vegetable/fruits exposed to oxygen (Constabel & Barbehenn, 2008) (Figure 2a). In the scientific community the interest in PPOs for the development of new strategies for preventing browning reactions for both fresh fruits and processed foods is thus increasing.

Plant PPO proteins have three domains: a chloroplast transit peptide (cTP) at the N-terminus, a dicopper center (CuA and CuB) and a C-terminal region (Figure 2c, 2d) (van Gelder et al., 1997). *Via* the twin arginine-dependent translocation pathway, the cTP regulates import into the thylakoid lumen (Koussevitzky et al., 2008). In the dicopper center there are two conserved copper-binding domains, each one composed by approximately fifty amino acids with three histidine residues that comprise the active site; the CuA and CuB are separated by a linker segment of 100 amino acid residues (Figure 2d) (van Gelder et al., 1997). The dicopper center is conserved but CuA domain is more variable; this variability may affect substrate preference. The C-terminal domain represents instead the protein region susceptible to proteolytic activity. Cleavage of this domain results in an increased activation of latent PPO (Robinson & Dry, 1992).

The high conserved Cu-binding domain of PPOs facilitates the isolation of PPO cDNAs from different Angiosperm species. This feature suggests the presence in plants of multiple, intronless, *PPO* genes. In tomato seven single-exon *PPO* genes were characterized (Newman et al., 1993), while five were found in potato (Thygesen et al., 1995). Globe artichoke contains seven intronless *PPO* genes (unpublished result: see Results of this thesis). However other *PPO* genes, with introns in the sequence, had been found in many plants, thus comparing sequences is not an efficient strategy to capture the whole diversity of *PPOs*. Whole genome sequencing projects help to identify all *PPO* genes in plants, and *in silico* analysis confirm the high variability in number and structure of the *PPO* family (Tran et al., 2012).

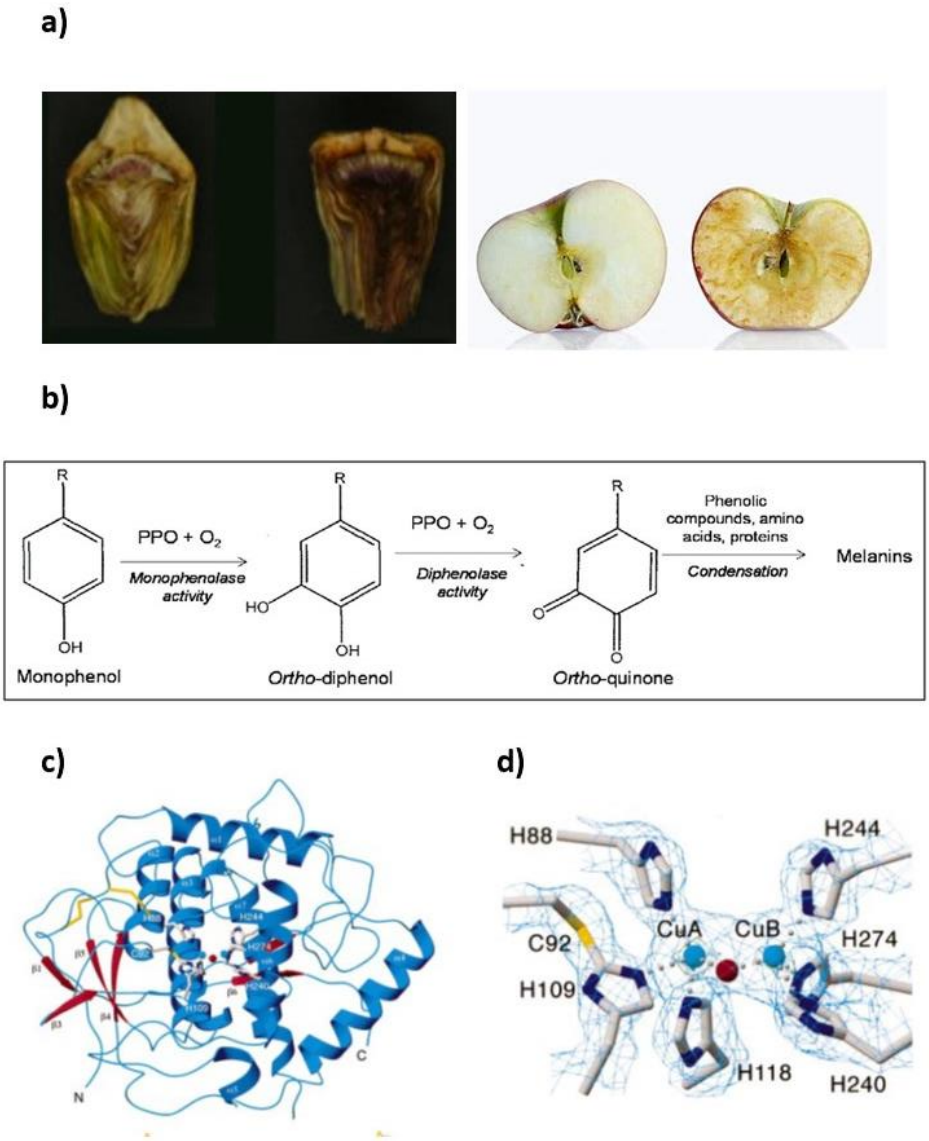


Figure 2. Illustration of PPOs-mediated browning and PPO's structure. (a) Pictures showing PPOs-induced browning effect in globe artichoke (left) and apple (right) after cutting (personal images). (b) Schematic representation of the enzymatic reaction mediated by PPOs responsible of the production of brown pigments in plant tissues exposed to oxygen (Taranto et al., 2017). (c) Front view of a PPO tertiary structure (Klabunde et al., 1998). (d) Representation of the central oxidized catalytic dinuclear copper site of PPOs (Klabunde et al., 1998).

The physiological roles of PPOs are not well known. Perhaps PPOs have function in plant defense because their expression is induced by herbivorous, pathogens and wounds. Most PPOs are localized in the chloroplast while their phenolic substrates are stored in the vacuole. This differential localization indicates that PPO enzymes can be in contact with their substrate only when a damage of the cell occurs, suggesting the defensive role of PPO in *plant*a. A publication reporting gene expression analysis of *PPO* genes in poplar (*Populus trichocarpa*) revealed a differential expression pattern of these genes following different plant treatments,

demonstrating that a small set of *PPOs* is upregulated by wounding, pathogen infections or methyl jasmonate (Tran & Constabel, 2011).

The PPO-generated quinones alkylate amino acids essential for the insect feeding, making them no longer available for the insect. In tomato's leaves *PPO* genes are wound-induced and regulated *via* the octadecanoid wound-signaling pathway. This mechanism acts as an anti-nutritive defense mechanism against insects (Constabel & Ryan, 1998). Studies conducted in other crops, from different families, showed that only in some species there is an activation of *PPO* induced by wounding or methyl-jasmonate, like tomato (*Lycopersicum esculentum*), hybrid poplar (*Populus thrichocarpa x deltoides*) and tobacco (*Nicotiana tabacum*) (Constabel & Ryan, 1998). Transgenic approaches are useful to study the defense activity of PPOs against insects, bacterial and fungi, through down-regulation or over-expression strategies. A potato's (*Solanum tuberosum L.*) *PPO* gene, posed under the control of the *cauliflower mosaic virus 35S* promoter, was introduced in tomato (cv. Moneymaker) obtaining a transgenic plant overexpressing this *PPO* gene (Li & Steffens, 2002). When the resistance against *Pseudomonas syringae* patovar *tomato* pathogen was tested in this plant, reduced disease symptoms were observed compared to wild type plants (Li & Steffens, 2002), thus confirming the defensive role of PPOs.

1.2.2 Anti-nutritional effect of PPOs

PPO-generated browning has a negative impact on the quality of a wide range of fruits and vegetables due to the decrease of the shelf life and of the nutritional properties (flavor, color, texture and nutrient values) of the product (Vamos-Vigyázó, 1981). The PPOs are involved in vegetables and fruits ripening, in fact they remain active in post-harvesting phases, causing negative effects on the product and resulting in minor acceptance from consumers. Within this framework, *PPO* genes have gained much interest in the scientist community as potential candidates for research investigations.

1.2.3 Strategies to reduce PPOs-mediated enzymatic browning

Browning reactions that occur in vegetable and fruit products can be divided into enzymatic or non-enzymatic reactions. The non-enzymatic reaction involves single or multiple compounds and does not include enzymes activity, like Maillard reaction, ascorbic acid oxidation and caramelization (Maillard, 1912). Differently, enzymatic reaction involves the activity of the polyphenol oxidase (PPO); this mechanism occurs in fruit and vegetable products during harvesting, storage and processing, negatively influencing the quality of the products (Figure 2a). Even mechanical and physical processes can aggravate the enzymatic browning, like cutting and peeling that expose the PPOs and the phenolic compounds to the atmospheric oxygen, with the subsequent oxidation of the substrates into corresponding

quinones. These quinones are polymerized forming a brown pigment called melanin (Queiroz et al., 2008) (Figure 2b).

To prevent PPO activation, the study of factors that influence their activation, including pH, presence of oxygen and concentration of phenol compounds, is necessary. These approaches for the prevention of enzymatic browning can be physical or chemical (Devece et al., 1999).

Physical methods include heat treatment or blanching which can reduce the enzymatic activity by inducing protein denaturation. Blanching can have negative effects on flavor, texture and color, indeed it is replaceable by elimination of oxygen to reduce *PPO's* activation using packaging films and inert gas, as nitrogen (N_2) or carbon dioxide (CO_2). Another way to store up the product consists into the immersion in sugar or salt solutions, to avoid contact with oxygen (Moon et al., 2020). In addition, it is possible to use lower temperatures to increase the shelf life of the product suppressing the activation of PPOs (Chaves & Zaritzky, 2018).

Chemical methods are based on the application of antioxidants, chelating agents or natural extracts. PPOs can be inhibited by lowering the pH using ascorbic acid, citric acid or glutathione (Zemel et al., 1990). At low concentrations, ascorbic acid and cysteine can act as competitor inhibitors. However, at higher concentration (>1,5%) ascorbic acid can reduce the quinone generated by PPOs, and cysteine reacts with quinone to give a colorless product (Ali et al., 2015). Another reducing agent is sulfate and its derivatives, which are irreversible inhibitors of PPOs (Sayavedra-Soto & Montgomery, 1986).

The food industry wants to meet the interests of the consumers being more sustainable by limiting the waste of food. For this reason, it is important to reduce the browning reactions, which are the main cause of food waste (Whitaker J. R. & Lee C. Y., 1995). Using sustainable inhibitors of PPO like food by-product extracts may represent a solution to meet these interests.

Since these industrial methods do not solve the problem but just slow down the process, the use of targeted genetic engineering represents the best strategy to overcome this limit. Indeed, the inactivation of *PPO* genes allows to suppress the browning without decreasing the phenolic concentration thus maintaining the nutritional value of the products. It may also increase the shelf life of the final products diminishing the food waste. Different examples of genetic engineering approaches applied to *PPO* genes will be discussed in the next section (see paragraph "Genetic engineering of *PPO* genes").

1.3 Plant genetic engineering: from conventional breeding to gene editing *via* CRISPR/Cas system with a focus on *PPO* genes

The oldest method for genetic improvement dates to eight/ten thousand years ago, when agriculture takes roots, with the selection of best seeds and plants to use in the next season for breeding new phenotypes. Domesticated plants were born in this way, choosing the best quality of the plant, a better pest resistance, a faster growth, a better taste of the product or a better production rate ("Pocket K No. 13 Conventional Plant Breeding", 2004). These differences derive from generic variations in species occurring naturally in the progeny.

The genetic mutations may have positive or negative effects on plants, or even neutral. They can occur naturally during cell division, can be caused by virus infections or ionizing radiations. Mutations can introduce small alterations, like a single nucleotide base pair deletion, insertion, and substitution, or large DNA fragment modifications, such as DNA translocations or inversions (Directorate-General for Research and Innovation & European Commission, 2017).

1.3.1 Conventional breeding techniques

The Conventional Breeding Techniques (CBTs) used by farmers and seed producers are simple selection, inter- and intra-species crossing, hybridization for vigorous, somatic hybridization, bridge cross, doubled haploids and polyploids induction, translocation breeding, mutation breeding and embryo rescue (Figure 3a, above). These techniques have been used for decades, however their application is laborious and time consuming and it is often associated with off-targeting, i.e. the occurrence of undesired mutations in off-target genomic sites (OTs). OTs can be eliminated by backcrossing although this process becomes difficult if OTs are located near the genomic trait carrying the desired mutation (Directorate-General for Research and Innovation & European Commission, 2017). Moreover, the backcross can lead to linkage drag, which is the transfer of deleterious genes linked to the gene of interest (Hou H. et al., 2014).

1.3.2 Established techniques of genetic modification

At the end of the 1970s the Established Techniques of Genetic Modification (ETGM) were developed (Figure 3a, below left); these techniques are based on the incorporation of genetic fragments into organisms allowing even interkingdom transfers, because sexual compatibility isn't required (National Research Council, 2004).

ETGM use recombinant DNA traits containing one or more genes of interest with appropriate promoter and terminator sequences, a selectable marker gene, an origin of replication (ORI) and a multiple cloning site, forming a so-called construct. This construct is

transferred into a microorganism's cell: the ORI allows the autonomous replication in the microorganism while the multiple cloning site is needed for the integration of additional desired DNA sequences (Directorate-General for Research and Innovation & European Commission, 2017).

Different methods can be used to deliver the construct into plant cells. These methods can be grouped into direct and indirect methods. Direct methods include electroporation of protoplasts, enzymatic treatment, polyethylene glycol (PEG), viral infection and particle bombardment (biolistic, or gene gun). Differently, indirect methods involve the use of a biological vector, e.g. *Agrobacterium tumefaciens* that has the ability to naturally infects plants and transfer a part of its DNA (transfer DNA, T-DNA) into the host genome. Based on this mechanism, it is possible to substitute a part of *A. tumefaciens* T-DNA with the gene of interest in order to deliver the desired trait into a plant cell (Directorate-General for Research and Innovation & European Commission, 2017).

1.3.3 New breeding techniques with a focus on the CRISPR/Cas system

The constantly growing knowledge in plant genomics permitted to develop new methods for inducing more precise gene mutation, i.e. the new breeding techniques (NBTs) (Figure 3a, below right). NBTs are divided into five groups based on the type of the modification induced. The Group 1 includes site specific mutagenesis techniques (meganucleases (Figure 3b), zinc finger nucleases (ZFNs) (Figure 3c), transcription activator-like effector nucleases (TALENs) (Figure 3d), oligonucleotide directed mutagenesis and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system (CRISPR/Cas) (Figure 3e)); the group 2 includes cisgenesis and intragenesis; the group 3 includes breeding with transgenic inducer lines (reverse breeding and RNA-dependent DNA methylation); the group 4 includes grafting techniques; the group 5 includes agro-infiltration techniques (agro-infiltration, agro-infection and floral dip). Among all these techniques, over the last decade those from group 1 (Figure 3b-3e) were certainly the most widely used in crop genetic improvement, due to their rapidity and high specificity in introducing the desired trait into a plant genome (Martínez-Fortún et al., 2017; Ran et al., 2017; Zhang et al., 2018).

ZFNs, TALENs and meganucleases act by recognizing specific DNA sequences through a DNA-protein binding mechanism with consequent introduction of double strand breaks (DSBs) that are fixed by the cell's endogenous repair systems, either by homologous recombination (HR) or non-homologous end joining (NHEJ), resulting in a mutation of the targeted genomic DNA sequences (Martínez-Fortún et al., 2017; Ran Y. et al., 2017; Zhang et al., 2018) (Figure 3f). Although innovative, these methodologies are based on the use of multiple nuclease monomers and sequence-specific DNA binding domains (Figure 3b-3d), thus requiring difficult design and cloning procedures. On the contrary, over the last decade the CRISPR/Cas system, which acts similarly to ZFNs, TALENs and meganucleases but involves a simple programmable

RNA-guided DNA endonuclease system (Jinek et al., 2012) (Figure 3e, 3f), has emerged as a powerful alternative to precisely and quickly insert the desired trait into a plant genome (Feng et al., 2013; Martínez-Fortún et al., 2017; Ran et al., 2017; Zhang et al., 2018).

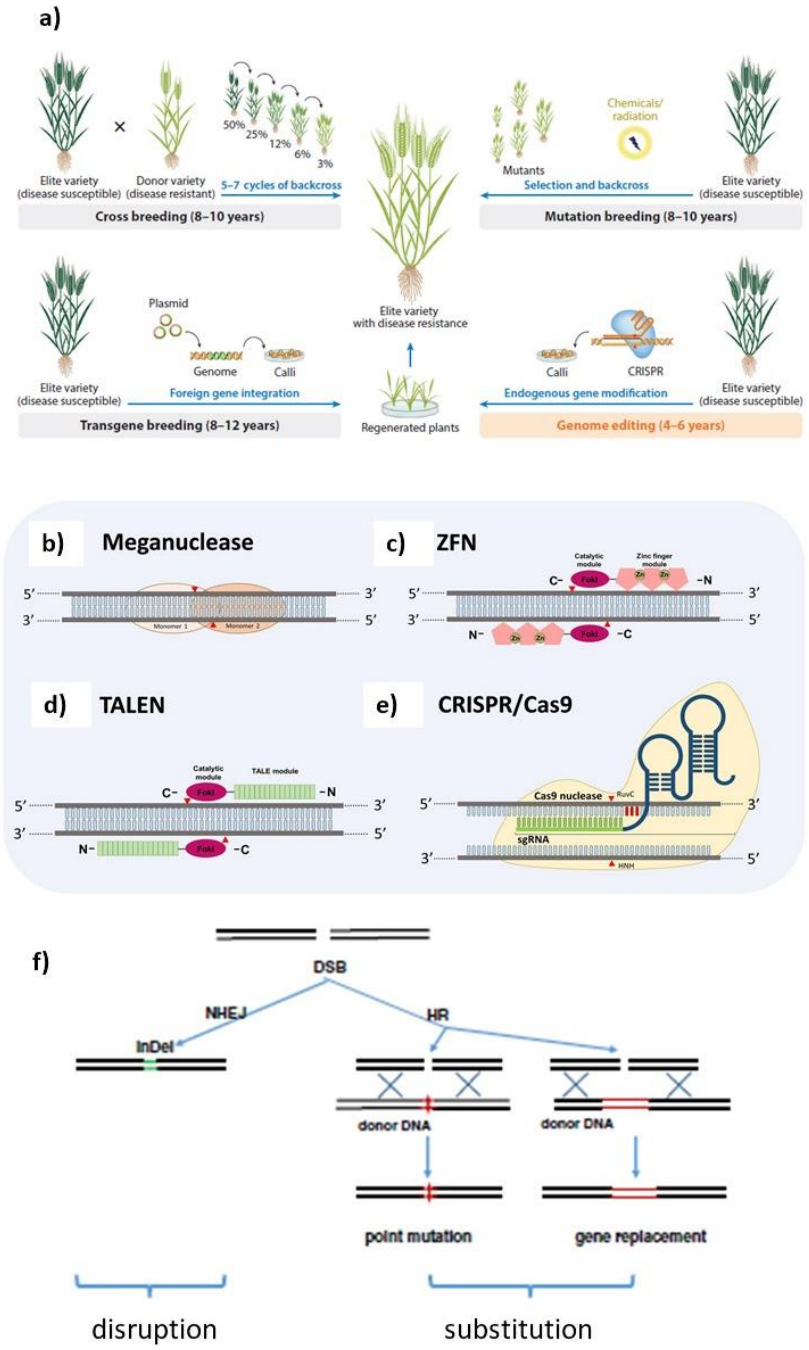


Figure 3. Overview on the genetic strategies used in crop improvement. (a) Illustration of different approaches used for plant genetic engineering with a focus on the corresponding developmental times required (Chen et al., 2019). (b-e) Schematic representation of structure and mechanism of action of the most commonly used NBTs: meganuclease (b), ZFN (c), TALEN (d) and CRISPR/Cas systems (e) (Romay & Bragard, 2017). (f) Schematic illustration of mutations introduced by NHEJ and HR during DSBs repairing (Chen et al., 2014).

The history of the CRISPR/Cas system

The origin of CRISPR/Cas system dates back to 1987, when an unusual repetitive DNA sequence, later called CRISPR, was observed in *Escherichia coli* (*E. coli*) during genetic analysis on phosphate metabolism (Ishino et al., 1987). In later years, similar DNA patterns were detected in other bacteria and by further studies of structural biology, comparative genomics and biochemistry, the whole process underpinning the CRISPR/Cas system was described (Ishino et al., 2018). In middle 2000s, Francisco J. M. Mojica and Christine Pourcel independently noticed that the spacer DNA sequences between the repeated CRISPR regions were homologous to bacteriophages, plasmids and prophages sequences (Mojica et al., 2005; Pourcel et al., 2005). This observation suggested the involvement of the CRISPR system in the prokaryotic immune defense by capturing fragment of foreign DNA to memorize invaders' genetic features. In parallel, other genomic studies identified different genes with endonuclease activity that are strictly associated with CRISPR sequences, defined as *Cas* (CRISPR-associated) genes (Jansen et al., 2002). Comparative genomic analyses thus proposed that CRISPR and Cas proteins act in synergy as a silencing machinery to guide disruption of invading nucleic acids (Makarova et al., 2006). In 2007, the entire mechanism of CRISPR/Cas immune system in bacteria and archea against viruses was described (Barrangou et al., 2007). In 2012, a milestone has been set in the field of application of the CRISPR/Cas system. Indeed, Jinek and colleagues successfully engineered (Figure 3e) and applied *in vitro* the CRISPR/Cas system to introduce DSBs in a subset of target DNA sequences (Jinek et al., 2012), highlighting the potentiality of this system for genome editing purposes. Finally, in 2013, the CRISPR/Cas system was successfully used for the first time to perform site-specific genome editing in mammalian cells (Cong et al., 2013) and model plants (Feng et al., 2013). From that moment on, the CRISPR/Cas system has become a routine practice for targeted gene editing.

Classification and structure of the CRISPR/Cas system

CRISPR/Cas system is categorized into Class 1 (multiprotein effector complexes) and Class 2 (single protein effector complexes), and into 6 types and 33 subtypes (Dugar et al., 2018). The most commonly used CRISPR/Cas system comes from *Streptococcus pyogenes* (*S. Pyogenes*) and is named CRISPR/SpCas9 (Figure 3e). This system belongs to the type II CRISPR system and comprises four genes: *SpCas9*, *Cas1*, *Cas2* and a CRISPR array *Csn2*. The latter is composed by one leader and numerous CRISPR units, each one formed by one spacer and one repeat coding respectively for small interfering CRISPR RNAs (crRNAs) and trans-activating CRISPR RNAs (tracrRNAs) (Wang & Li, 2021). When the CRISPR array is transcribed and processed, crRNAs and tracrRNAs interact with each other to form an RNA-RNA hybrid that guide the Cas9 endonuclease to the target DNA sequence (Figure 3e).

Cas9 is a monomeric nuclease containing a central nucleic acid recognition domain (REC) and two endonuclease domains: the histidine-asparagine-histidine (HNH) domain that

nicks in the crRNA complementary strand and the RuvC domain that nicks in the non-complementary strand (Jinek et al., 2012) (Figure 3e). The DNA sequence targeted by the crRNA, to be recognized, must present a protospacer adjacent motif (PAM) (Figure 3e). In general, PAM is a 3-6 nucleotides long conserved sequence downstream the DNA sequence targeted by the crRNA and is CRISPR/Cas-type specific. For CRISPR/SpCas9 system, a 5'-NGG-3' PAM sequence is required (Mojica et al., 2009).

Mechanism of action of the CRISPR/spCas9 system in the prokaryotic immune defense

The prokaryotic immune defense mediated by the CRISPR/Cas9 system can be described by a three-steps model (Figure 4). The first step is the adaptation, which consists in the excision of spacer DNA sequences from foreign DNA and their subsequent integration into the CRISPR/SpCas9 locus (Wang & Li, 2021). In detail, following transcription and processing of the CRISPR locus, the adaptation complex, formed by Cas1, Cas2 and Cas9, recognizes the PAM site and cleaves out the adjacent DNA sequence (protospacer) that is then inserted as a spacer between two repeats into the CRISPR array. The second step consists in the expression of CRISPR array into a long transcript (functional non-coding RNA) precursor of crRNA, which base pairs with tracrRNA (Wang & Li, 2021). This complex is cleaved by RNA polymerase III associated to SpCas9 protein with the consequent release of the mature crRNA, formed by a 20 nucleotides long spacer and a 22 nucleotides long repeat. Finally, the third step consists in the interference (Wang & Li, 2021): The tracrRNA and the crRNA guide spCas9 to the target DNA sequence allowing the cleavage of foreign DNA.

Optimization and engineering of the CRISPR/spCas9 system for gene editing purposes

Ten years ago, by observing how the natural CRISPR/Cas system works in bacteria and archea, an optimized CRISPR/SpCas9 system usable for programmable site-specific genome editing was developed. In detail, to facilitate the artificial gene editing, the dual crRNA-tracrRNA complex was replaced by a single chimeric RNA sequence called single guide RNA (sgRNA) composed by a 20-nucleotide spacer (which determines the genomic target) and a scaffold sequence necessary to bind the Cas protein (Jinek et al., 2012) (Figure 3e, 4a).

Besides the efficiency and simplicity in introducing stable mutations, one of the advantages of the CRISPR/Cas system is represented by its ability to be programmable for targeting a multitude of DNA sequences. Indeed, the sgRNA is short in length and can be easily engineerable by simply modifying the 20 nucleotides long gRNA sequence. Several sgRNAs can thus be used in a multiplexed CRISPR/SpCas9 system to simultaneously target different DNA sequences (Hassan et al., 2021; Ma & Liu, 2016). On the other side, a limit of this system is represented by the need of the PAM, which restricts the number of targets in a given DNA sequence. In addition, based on the gRNA similarity with the sequence in object, the CRISPR/spCas9 system may also be associated with unwanted OT activity (Xu et al., 2019). To

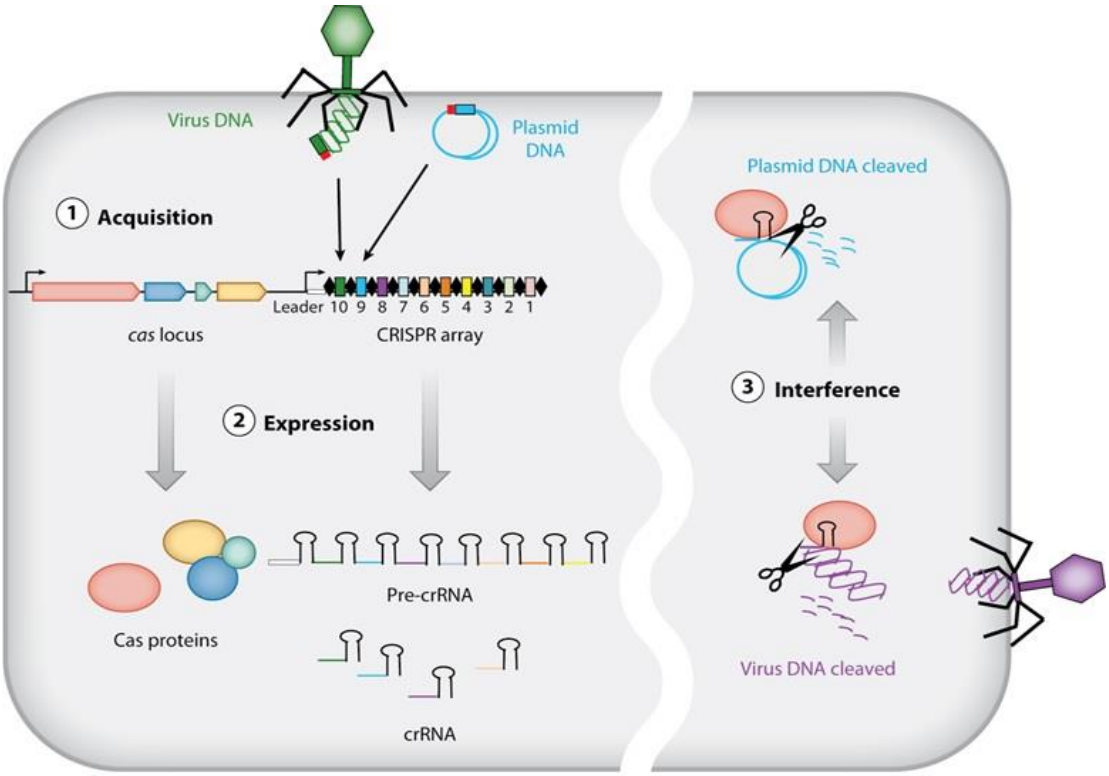


Figure 4. **Schematic illustration of the three-steps model describing the mechanism of action of CRISPR/Cas9 system in the prokaryotic immune defense.** (Bhaya et al., 2011)

evaluate possible OTs, bioinformatic programs are used, allowing customers to design the best gRNA. Two methods have been developed to minimize the OTs: the Cas9-nuclease fusion system and the double nickase. The latter increases the system specificity by using two sgRNAs nicking in two sites of the same target sequence (Figure 5b). Indeed, this system permits to operate two single strand breaks, lowering the OTs rate, because if an OT single strand break occurs the cell can repair it with high fidelity (Ran F.A. et al., 2013). Differently, the Cas9-nuclease fusion system uses two sgRNAs and a catalytically inactivated Cas9 (deadCas9, dCas9) (Figure 5c). dCas9 lacks the nuclease activity and is fused with FokI, a bipartite restriction endonuclease (fCas9). Two associated monomers of fCas9 work for the DNA cleavage by binding the target sites 15 to 25 base pairs apart. With this mechanism of action, fCas9 doubles the number of targeted bases thus increasing the specificity of the system and lowering the OT events (Guilinger et al., 2014).

The application of dCas9 gives advantages not only to reduce CRISPR-associated OT activity but also to increase the ways for applying targeted gene manipulation. Indeed, over the past few years, several studies have made use of multiple re-engineered CRISPR/dCas9 systems to: (i) modulate the expression of genes through recruitment of transcriptional effector domains without introducing DNA-damaging mutations (Figure 5d); (ii) recruit modifying enzymes and reporter proteins to DNA target sites (Figure 5e, 5f); (iii) induce gene

activation and repression (Figure 5d, 5e); (iv) perform epigenome editing (Figure 5f), as recently reviewed by Moradpour & Abdulah (2020). Another important application of dCas9 is the base editing: dCas9 can be fused with a cytidine deaminase (to obtain a cytidine base editing, CBE) or adenosine deaminase (for an adenine base editing, ABE). With CBE a C-T to T-A single-base substitution is applied, while ABE results in A-T to G-C substitution (Bharat et al., 2020).

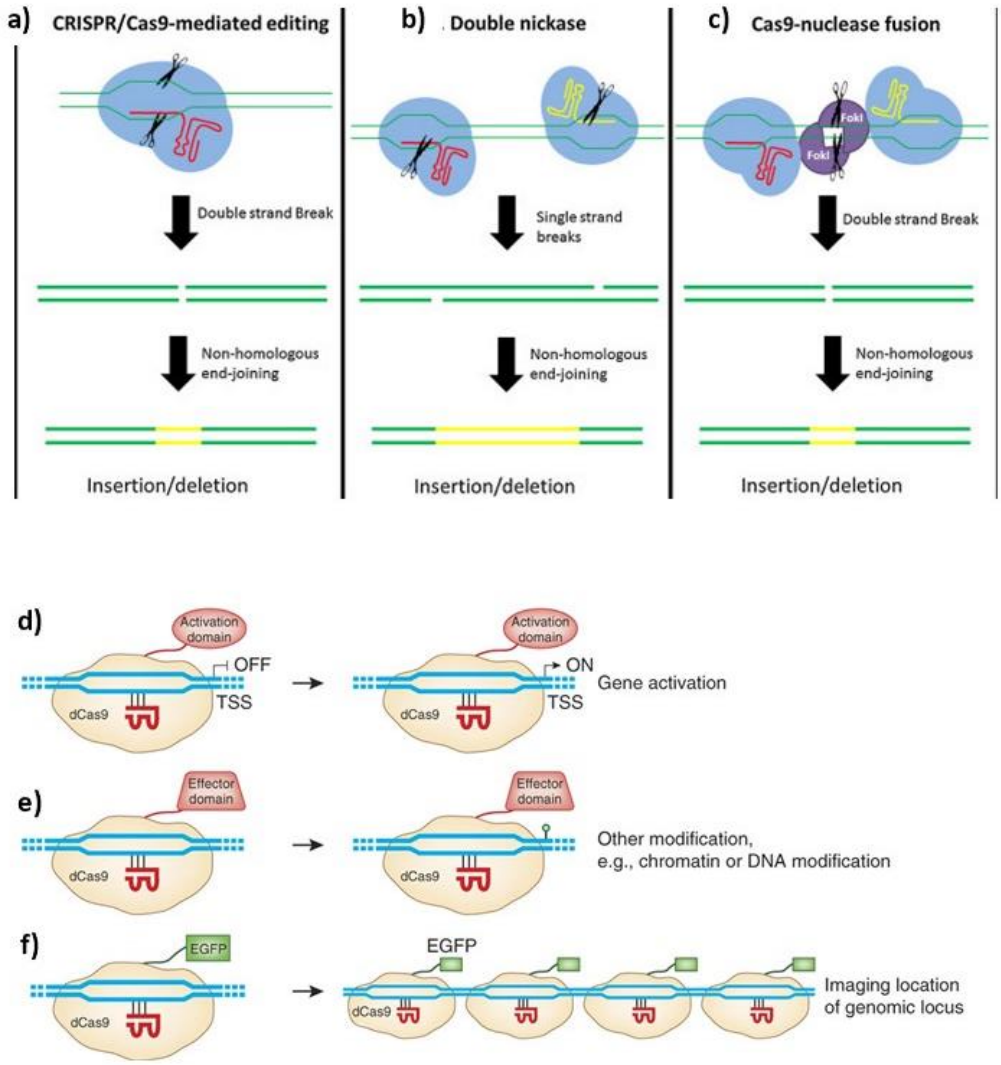


Figure 5. Overview on the different engineered CRISPR/Cas9 systems used in plant molecular studies and crop improvement programs. (a) CRISPR /Cas9 system. (b) double nickase. (c) Cas9-nuclease fusion. (d) CRISPR/Cas9-mediated activation or repression of gene expression. (e) CRISPR/Cas9-mediated genome and epigenome modification. (f) CRISPR/Cas9-mediated recruitment of reporter proteins to DNA target sites. (a, b, c: Schaeffer & Nakata, 2015) (d, e, f: Sander & Joung, 2014)

1.3.4 Genetic engineering of *PPO* genes

Over the last decade, different methods have been used to knock-out *PPO* genes, or reduce their expression, with the aim of preventing enzymatic browning in crops, thus increasing the shelf life of the product. To date, different examples have been reported for species of agronomic interest like apple, potato, eggplant and mushroom.

A well-known event of *PPO* mutagenesis is represented by the Artic® apple (Stowe & Dhingra, 2020), which does not brown after cutting, offering fruit freshness longer. This apple was obtained by targeting *MdPPO2* gene using an RNA interference (RNAi) approach, i.e. a post-transcriptional gene silencing (PTGS) methodology in which the messenger RNA (mRNA) targeted is eliminated thus resulting in the interruption of the corresponding protein translation. The generated transgenic apple was analyzed over years to control the occurrence of any undesired mutation and no differences have been identified with respect to the parental cultivar (Stowe & Dhingra, 2020).

Another example is the study conducted by Chi M. and colleagues in 2014, that aimed at reducing the PPO-mediated enzymatic browning in potato (Chi M. et al., 2014). The authors through a gene expression analysis, observed that the nine *PPO* genes (*StuPPO1-9*) comprised into the potato genome differentially contribute to the browning. A set of transformation vectors was thus assembled, based on the artificial micro RNAs (amiRNAs) technology, to silence different combinations of *StuPPOs*. The greater reduction of the enzymatic browning was observed when *StuPPO1-4* were silenced in tandem. In potato, knock-out of *StuPPOs* was also conducted through CRISPR/Cas9-mediated mutagenesis (González M. et al., 2021). In detail, CRISPR/Cas9 components were designed to simultaneously target 2 sites of the *StuPPO2* gene sequence, and delivered by either *A. tumefaciens* and as ribonucleoprotein complexes to plant protoplasts. The authors concluded that the efficiency of the CRISPR/Cas9-mediated mutagenesis in potato strictly depends on the delivery strategy used.

Recently, an important example of CRISPR/Cas9-mediated *PPO* gene silencing was obtained in eggplant (*Solanum melongena* L.) (Maioli et al., 2020). The eggplant genome contains ten *PPOs* (*SmelPPO1-10*) and three of them are highly expressed in the fruit after cutting (*SmelPPO4*, *SmelPPO5* and *SmelPPO6*). A conserved region of these three *PPOs* was exploited by the authors to design a CRISPR/Cas9 system carrying a single gRNA targeting this region. Following *A. tumefaciens*-mediated transformation of plant cotyledons, stably edited plantlets were regenerated, with no OT mutations highlighted. Thus, in these plants the introduced mutations in *SmelPPO4-6* were associated with a reduced PPO activity and browning of the berry after cutting.

Another interesting and successful gene-editing example is the case of the common mushroom (*Agaricus bisporus*) that was modified using CRISPR/Cas9 system obtaining the deletion of few bases of the gene of one *PPO* resulting in a knockout; it showed the reduction

of enzymatic browning of 30% (Waltz E., 2016). The genetically modified mushroom can be cultivated and sold in US because it doesn't contain foreign DNA from viruses or bacteria.

On this basis, the state-of-the-art on genetic engineering of plant *PPO*s highlights good expectations for a broad application of this strategy in other agronomically important plant species showing PPO-mediated browning, such as globe artichoke.

1.4 State-of-the-art on *in vitro* culturing and transformation procedures in globe artichoke species

1.4.1 Plant micropropagation

Globe artichoke has been traditionally propagated through offshoots, stumps or "ovoli", however these procedures lead to the transmission of diseases and produce low plants' heterogeneity and rate of multiplication (Pecaut & Dumas de Vaulx, 1983). On the contrary, the best way to perform a safe and rapid propagation of globe artichoke is represented by *in vitro* culturing (Figure 6).

Micropropagation is an *in vitro* culture technique which accelerates vegetative propagation of plants, by producing large numbers of individuals with the same genotype (clones). Due to its rapidity in the production of numerous disease-free clonal plants, this technique is often linked to the generation of transgenic plants. Four steps are required to settle micropropagation: (i) the settlement of aseptic *in vitro* culture, (ii) shoots multiplication, (iii) rooting and (iv) acclimatation. There are many advantages using micropropagation: large scale propagation, disease-free material, homogeneity of plant material, conservation of germplasm and independence from seasonal cycle (Comino C. et al., 2019).

In 1973, De Leo and Greco applied for the first time micropropagation to globe artichoke, elaborating culture conditions adapted to globe artichoke necessities. Subsequently several reports have been conducted to improve micropropagation efficiency using different plant tissues and different culture conditions, like culture media or growth regulators (Comino C. et al., 2019). In globe artichoke, the first step to start an *in vitro* culture consists in the sterilization of the explants in order to remove fungal and bacterial contaminations (Husain & Anis, 2009). Plants collected from field must be washed with water and then in antioxidant solution to avoid activation of *PPO* genes during sterilization. If this step is skipped the tissue browning can adversely affect the *in vitro* growth (Comino C. et al., 2019). Following sterilization, plant explants are moved to a so-called propagation medium whose composition must be precise and adapted to the type of plant tissue used. In general, a nutrient medium for globe artichoke propagation or multiplication contains macro and micro elements, organic supplements, vitamins, carbon source, growth regulators (hormones) and gelling agent to solidify the mixture. A largely used medium for plant micropropagation is represented by the Murashige and Skoog substrate (1962). Depending on the aim of the culture and on the cultivar used, it is possible to change growth regulators to optimize the experiment (Comino C. et al., 2019).

Figure 6. Picture showing a globe artichoke plantlet maintained *in vitro* onto a propagation medium. (Personal image)

1.4.2 Plant regeneration

Regeneration is often a limiting step in biotechnological approaches. It is essential to have an established, reproducible and efficient regeneration method before starting any biotechnological strategy that aims at the genetic improvement of crops.

The *in vitro* tissue culture stands on the totipotency of plants, defined as the capacity of cells and tissues to develop an entire plant when stimulated with the appropriate growth regulators. The first step of regeneration is morphogenesis, which can imply somatic embryogenesis or shoot organogenesis, and subsequently root organogenesis takes place. These pathways can occur directly, or indirectly with an intermediate callus stage (Gamborg OL & Philips GC, 1996). The morphogenesis is influenced by several factors like genotype, explant source, physiological stage and growth regulators.

To date, several reports described efficient protocols for *in vitro* micropropagation of globe artichoke. On the contrary, procedures for biotechnological approaches requiring plant regeneration are still lacking due to the recalcitrance of this plant species to morphogenesis. For regeneration, different explants can be used, although it is thought that young tissues (like capitulum) have more regeneration capability (Comino C. et al., 2019). Nevertheless, no successful experiment has been published to date.

Indeed, only one example of indirect shoot organogenesis has been observed by the use of globe artichoke immature buds' as starting explants. In detail, from callus-like structures a *de novo* differentiation of shoot primordia was obtained (Ricardo J. Ordas, 1990). Based on this information, a outstanding limit exists for globe artichoke research, especially in terms of application of those advanced biotechnological approaches which require this essential plant regeneration step.

1.4.3 Plant genetic transformation

Genetic transformation is an important tool for the agronomical and technological improvement of crops. A bottleneck in applying genetic transformation in globe artichoke is due to its recalcitrance to *in vitro* regeneration and the consequent unavailability of efficient genetic transformation protocols.

The first example of genetic transformation in globe artichoke dates back to the 1990s, when Gonzalès and Kchouk successfully obtained transgenic calli from leaf explants transformed with *A. tumefaciens* containing the β-*glucuronidase* (*GUS*) reporter gene system and the *neomycin phosphotransferase* (*NPTII*) gene (Gonzales & Kchouk, 1994; Kchouk et al., 1997). Similar results were observed when the same authors applied particle bombardment to axillary buds with the aim of bypassing tissue culture-related regeneration limitations (Gonzales & Kchouk, 1994). Menin and colleagues also genetically transformed globe artichoke leaf explants, of Romanesco type, using an *A. tumefaciens* containing a binary vector carrying the *GUS* reporter gene (Menin et al., 2012). Leaf explants generated calli stably transformed, however, subsequently no shoot was regenerated. More recently, a successful transformation of globe artichoke was performed by the use of *A. rhizogenes* as innovative strategy for the production and isolation of inulin for food industry scopes (Meneghini, 2016). In 2021 Paolo Dario et al. set up a genetic transformation of cardoon cells using *A. tumefaciens* for the overexpression of a transcription factor of *A. thaliana* (MYB4), a repressor of lignin/flavonoid biosynthesis. The cell line obtained can grow faster and has a major accessibility to the metabolic compounds due to less lignin content.

1.4.4 Genetic tools for optimizing plant transformation detection and regeneration efficiency

Before starting any genetic engineering procedure, it is important to previously evaluate the state-of-the-art on the biotechnological methodologies applied to the species of interest and, if necessary, to set up preliminary experiments for choosing the best one. When efficient transformation and regeneration protocols are unavailable, different strategies can be used to help, on a side, the detection of a successful transformation and, on the other side, the improvement of regeneration efficiency. These strategies are based on the use of a multitude of genes which can be delivered via direct and indirect transformation methods to the plant system and function as markers or developmental regulators. In this regard, a brief overview of the most commonly used genes, with their function and application to the plant system is reported below.

Reporter genes for plant transformation detection

Examples of the most common reporter genes used to evaluate the efficiency of the delivery of transformation DNA sequences into a plant cell are those coding for the green fluorescent protein (GFP) and the GUS protein.

The GFP is a 27 KDa protein, discovered in jellyfish *Aequorea Victo*ria, which becomes easily detectable by emitting in green spectrum (maximum peak at 505 nm) when excited by blue/ultraviolet lights (maximum absorbances at 395 nm and 475 nm) (Stewart, 2001) (Figure 7a, 7b). This gene is also widely used in plant molecular studies to facilitate the detection of plant transformants in which the construct of interest has been successfully delivered (Leffel et al., 1997; Yuan et al., 2021).

The *GUS* reporter gene, isolated from *E. coli* (Jefferson et al., 1986, 1987), is vastly used in plant molecular research for histochemical assays, even in globe artichoke species (Gonzales & Kchouk, 1994; Kchouk et al., 1997; Menin et al., 2012). The system is based on the use of the substrate 5-bromo-4-chloro-3-insolyl-ß-D-glucoronide (X-Gluc) which is hydrolyzed by GUS protein producing a colorless intermediate, subsequently dimerized into the 5,5'-dibromo-4,4'-dichloro-indigo (diX-indigo) (Figure 7c). This final product can be clearly observed as a blue precipitate under light microscopy (Guivarc'h et al., 1996) (Figure 7d).

Visual marker genes for plant transformation detection

Widely used visual marker genes for detection of the occurred transformation are the NPTII protein, an exogenus marker, and the endogenous Phytoene desaturase protein.

The NPTII gene, sourced from tn5 transposon of *E. coli* (Berg et al., 1975; Gritz & Davies, 1983), confers transgenic plants resistance to antibiotics like kanamycin monosulphate. Kanamycin normally inhibits protein synthesis in plants by binding to ribosomes resulting in bleaching of plants. However, in the transgenic plants, the encoded enzyme, NPTII, catalyzes the phosphorylation of kanamycin which becomes inactive, thus protecting the plants from bleaching. This enables transgenic plants to grow in the presence of this antibiotic thus helping the detection of positive transformants. Since its first application for biotechnological purposes, NPTII has been routinely used in plant genetic engineering studies (Miki & McHugh, 2004; Rosellini, 2011).

The *Phytoene desaturase* (*PDS*) is probably the most common plant visual marker gene used in genetic engineering studies, due to its easy of detection. Being *PDS* involved in the carotenoid biosynthesis (Figure 8a), its inactivation leads to an albino plant phenotype clearly visible by simple macroscopic observations (Figure 8b) (Koschmieder et al., 2017). In the era of gene editing, the strategy to mutate and knock-out the *PDS* gene has been widely applied to quickly demonstrate the feasibility of transformation and gene editing since its mutation

causes photobleaching and albino phenotype (Hooghvorst et al., 2019; Komatsu et al., 2019; Lu & Tian, 2022; Pavese et al., 2021).

Developmental regulators for increasing plant regeneration efficiency

In planta, the aerial organs of the plants are generated from the shoot apical meristem (SAM) while the grounded organs by the root apical meristem (RAM). The ability of SAM and RAM in inducing the production of new organs is due to the presence in these two regions of a population of pluripotent cells (stem cells), whose developmental state fate is precisely maintained by an equilibrium of a multitude of transcriptional factors throughout the entire life cycle (Sharma et al., 2003).

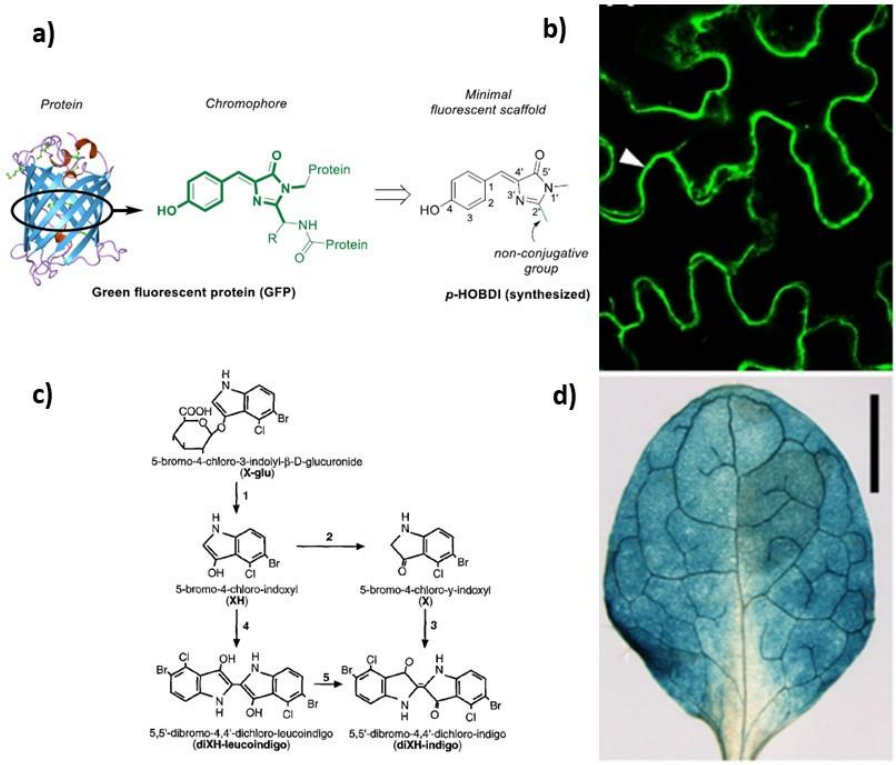


Figure 7. Most commonly reporter genes used in plant molecular research. (a) Structure of GFP chromophore (Jancsó et al., 2019). (b) Example of subcellular imaging of GFP-targeted constructs expressed in *Nicotiana benthamiana* agroinfiltrated leaves (Moglia et al., 2016). (c) Chemical reaction occurring in GUS assay (Guivarc'h et al., 1996). (d) Histochemical GUS assay of *A. thaliana* leaf transformed with a construct carrying a GUS reporter gene system (Pompili et al., 2020).

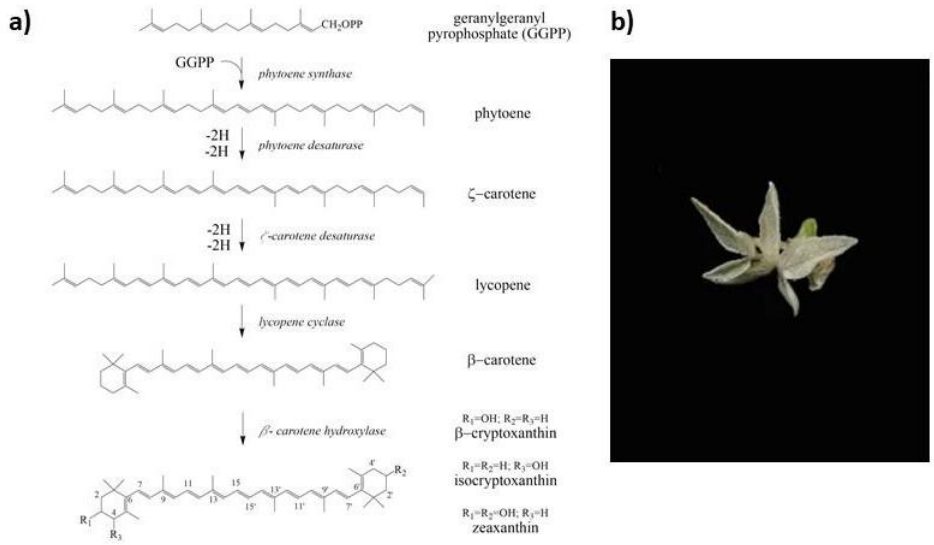


Figure 8. *PDS* visual marker gene. (a) Schematic illustration of plant carotenoid biosynthesis (Bautista et al., 2005). (b) Example of a *PDS* loss-of-function mutant in soybean showing an albino phenotype (Lu & Tian, 2022).

With regard to the SAM, four different regions can be observed: (i) three layers of cells (L1, L2 and L3 from the outmost to the innermost), (ii) the organizing center (OC), (iii) the Central cone (CZ) and (iv) two peripheral zones (PZ) (Figure 9) (Lopes L. et al., 2021). A distinct transcriptional profile for each region leads the organization of meristems.

WUSCHEL (WUS) is a homeodomain transcription factor produced by the cells of OC, able to migrate and stimulate the expression of CLAVATA 3 (CLV 3) in the CZ (Sharma et al., 2003). An equilibrium of WUS and CLV3 is established to maintain stem cell niche (Sharma et al., 2003). SHOOT MERISTEMLESS (STM) is another factor that acts in equilibrium with WUS to modulate the maintenance of undifferentiated cells. Indeed, STM mRNA accumulates in CZ and PZ, but lacks in organ primordia (Gallois et al., 2002).

Another important gene is the *isopentenyl transferase* (*IPT*), encoding the first enzyme involved in the cytokinin (CK) biosynthesis pathway (Sakakibara, 2005). The equilibrium of auxin and CK controls the activity of the apical meristems and CK are needed to maintain the stem cells in the SAM (Barton, 2010).

Last but not least, BABY BOOM (BBM) is an APETALA2 FAMILY/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (AP2/ERF) involved in plant embryogenesis (Jha & Kumar, 2018). In 2009, Deng and colleagues induced the overexpression of *BBM* gene in *Populus tomentosa*, resulting in somatic embryos developed from white calli which subsequently evolved into new plantlets.

Due to their functions, genes described above have potentiality in a view of application for biotechnological purposes. In last years, an important study successfully used these genes to generate gene editing through a *de novo* induction of meristems in different model plants and crop species (Maher et al., 2019).

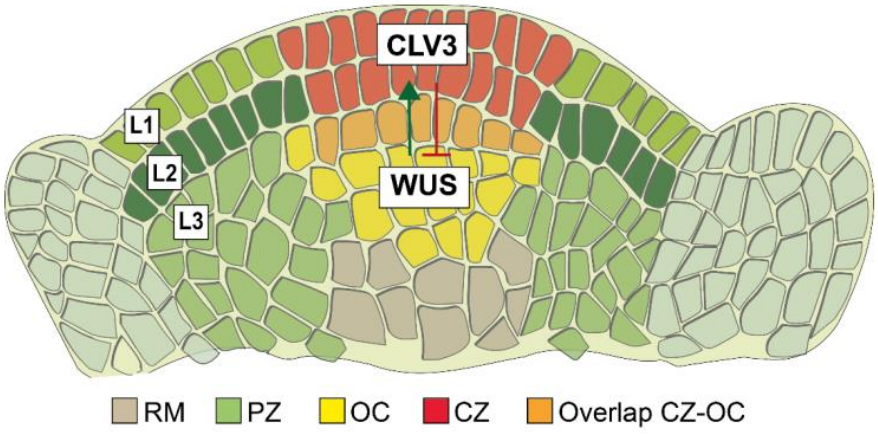


Figure 9. Schematic representation of tissue regions observed in a shoot apical meristem. (Lara Lopes et al., 2021)

1.5 Aim of the thesis

Since 2021, the University of Turin, and in particular the Unit of Plant Genetics Agriculture, has been conducting research activity within a big national research project named BIOTECH. This project comprises 11 subprojects dedicated to the application of new biotechnologies for the genetic improvement of different relevant species for the Italian agri-food system, such as vine, olive, apricot, peach, cherry, apple, pear, tomato, eggplant, wheat, poplar and globe artichoke. In detail, the Unit of Plant Genetics (where this thesis was conducted) has been enrolled as principal investigator within the QUALIMEC subproject, that aims at the improvement of qualitative properties in globe artichoke through gene editing. This thesis is part of this project and represents an initial part of this long-term goal to genetically improve globe artichoke species.

Globe artichoke has a worldwide economic impact for production of both primary and derived products, and Italy represents its main producer. An adverse characteristic of globe artichoke is the browning effect emerging after wounding, which decreases the shelf life and the quality of the final product. The browning is due to an enzymatic reaction catalyzed by the activity of PPOs, a multi-protein family which uses molecular oxygen to oxidize phenols to corresponding quinones, resulting in colored pigment formation in plant tissues. Despite their role in browning reactions is known, functions of *PPO* genes are still poorly clarified, especially in agronomically important plant species such as globe artichoke. More efforts are thus required to better characterize this gene family with the aim of setting up suitable gene editing strategies for PPO inactivation with the minimum trade-off for the plant.

Within this framework, the first step of this thesis will be the study of globe artichoke *PPO* genes and their corresponding promoter sequences through *in silico* and bioinformatic analyses in order to obtain information regarding their genomic features, transcriptional regulation and allele variations across four agronomically important globe artichoke varietal types (Spinoso di Palermo, Violetto di Sicilia, Violetto di Toscana and Romanesco C3).

Subsequently, with regard to the Violetto di Toscana varietal types selected for its high browning effects, a gene expression analysis by real time quantitative polymerase chain reaction (RTqPCR) will be conducted to experimentally investigate the transcriptional regulation of the identified *PPOs*, in different plant tissues and at different time points after wounding. This analysis will potentially promote the identification of those *PPOs* that are associated with browning of tissues, thus representing good candidates for a further gene editing approach.

Indeed, by using the Spinoso di Sardegna and Romanesco C3 varietal types for which efficient *in vitro* maintenance protocols are available, first steps towards the application of gene editing will be made. *PPOs* expression level will be evaluated at different callogenesis

conditions to acquire additional information regarding *PPOs* involvement in browning of plant tissues. Simultaneously, due to globe artichoke recalcitrance to *in vitro* regeneration, transformation and organogenesis efficiency will be tested by pilot experiments in different plant tissues using the ectopic expression of reporter genes and developmental regulators delivered *via A. tumefaciens*. In parallel, on the basis of all the acquired information, protocols for designing and assembling gene editing vectors for visual markers- and *PPOs*-inactivation will be performed. Vectors assembled will be used in additional *A. tumefaciens*-mediated gene transfer experiments.

2. MATERIALS AND METHODS

2.1 Bioinformatic resources and *in silico* analysis

2.1.1 Identification and structural characterization of PPOs

To identify all PPOs in *C. cardunculus* var. *scolymus*, PPOs amino acidic sequences previously annotated in the v.1.0 proteome (Scaglione et al., 2016) and available in the Globe Artichoke Genome Database (https://www.artichokegenome.unito.it) were used for a BLASTp analysis (https://blastp.ncbi.nlm.nih.gov) against the v.2.0 globe artichoke reference proteome (Acquadro et al., 2020), using an e-value threshold of $1e^{-5}$. The identified v.2.0 PPO protein sequences were downloaded. Based on their annotation, the corresponding gene and promoter sequences (1 Kbp upstream of the translation start site) were also isolated and retrieved from the v.2.0 globe artichoke reference genome (Acquadro et al., 2020) available at the Artichoke Genome Database.

The identified PPO protein sequences were analyzed using the Pfam software (https://pfam.xfam.org/) to predict protein structure domains. Instead, *PPO* genomic sequences were analyzed with the Wormweb software (https://wormweb.org/exonintron) to graphically generate a *PPO*-specific exons-introns profile.

2.1.2 Phylogenetic analysis of PPOs

The v.2.0 PPO protein sequences identified were used in a BLASTp search to find homologous in the non-redundant protein sequences NCBI database of 10 *Asteraceae* species (*Taraxacum officinale, Tanacetum cinerariifolium, Lactuca sativa, Heliantus annuus, Gerbera jamesonii, Cynara cardunculus* subsp. *Cardunculus, Dahlia pinnata, Mikania micrantha, Lactuca saligna, Erigeron canadensis*) and 3 *Solanaceae* species (*Solanum tuberosum, Solanum melongena, Solanum lycopersicum*), using an e-value cut-off of $1e^{-5}$. PPO protein sequences were downloaded and aligned to v.2.0 PPO sequences with the Clustal omega multiple alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/), using default parameters. The aligned PPOs were used to construct a phylogenetic tree with the Consurf program (https://consurf.tau.ac.il/overview.php) by applying a Neighbor joining algorithm (Saitou & Nei, 1987) as implemented in the Rate4Site program (Pupko et al., 2002).

2.1.3 *In silico* search of transcription factor binding sites in the promoter sequences of *PPOs*

To detect putative transcription factor binding sites (TFBSs) and corresponding transcription factors (TFs) involved in the regulation of *PPOs* expression, *PPO* promoter sequences (1 Kbp upstream of the ATG translation start codon) were examined by the "promoter analysis tool" of PlantPAN 2.0 (https://plantpan2.itps.ncku.edu.tw/promoter.php). TFBSs calling was performed against the *A. thaliana* reference database by considering: (i) a similarity score set to 0.95; (ii) only the coding strand comprising the *PPO* sequence. For each TFBSs, corresponding TFs identified were investigated by the PlantTFDB 5.0 database (http://planttfdb.gao-lab.org/) in order to acquire information regarding their biological function.

2.1.4 SNP/Indel analysis in genomic sequences of *PPOs*

The SNP/Indel analysis was conducted by mapping the *PPO* sequences (fastq) of Violetto di Sicilia, Violetto di Toscana, Spinoso di Palermo and Romanesco C3 genotypes to the v.2.0 reference genome, using a Burrows-Wheeler Aligner program (BWA) with default parameters. The SNP/Indel calling was conducted on the BAM files using Samtools mpileup with default parameters except for: (i) filter multimapping events (-q>1) and (ii) minimum mapping quality (Q=20). A variant call format (vcf) file was produced. SnpEff software was subsequently used to annotate allelic variants identified and to evaluate their impact on the protein function.

2.2 Molecular analysis of *PPO* family

2.2.1 Plant material, growth condition and sampling

Three different *C. cardunculus var. scolymus* varietal types were used for experimental analyses: "Violetto di Toscana", "Spinoso di Sardegna" and "Romanesco C3".

The Violetto di Toscana varietal type was used to analyze the expression level of *PPO* genes in different tissues (external bracts, internal bracts and receptacle of primary and secondary capitulum, leaves and stems) and at different time points after cutting. Plant material was collected by 1-year-old plants grown in field conditions at DISAFA (University of Turin, Carmagnola, TO, Italy). For capitulum sampling, capitulum was collected and vertically cut in 4 slices. Two slices were used for sampling (Figure 10a). From 1 slice, a ~3 centimeters cut-flanking section of external and internal bracts and receptacle was immediately sampled and frozen in liquid nitrogen, representing sample at time zero (T0) in the analysis (Figure 10a, left). From the other slice, the same tissues were sampled 15 minutes after cutting (T15) (Figure 10a, right). Sections of leaves and stems were collected only for T0 analysis. For each tissue, 3 samples (1 sample/plant) were collected. All samples were stored at -80°C before being used in further RNA extraction procedure.

The Spinoso di Sardegna varietal type was used *in vitro*, on a side, to evaluate gene expression level of *PPO* genes in different callus morphologies (Figure 10b) and, on the other side, to perform genetic transformation experiments. Plant material, i.e. *in vitro* propagated plantlets and SAMs, was kindly provided by the Agenzia Regionale Per La Ricerca In Agricoltura (AGRIS) (Cagliari, Italy). All plant material was maintained in *in vitro* pots using an in-house propagation medium (4,4 g L^{-1} of MS with vitamins, 30 g L^{-1} of sucrose, 0,5 mg L^{-1} of benzyl-aminopurine (BAP) and 7 g L^{-1} of plant agar), which was refreshed every 4 weeks. *In vitro* culturing was conducted at growth chamber conditions (24±1 °C and a photoperiod of 16 hours of light and 8 hours of darkness). In detail, for callogenesis induction, 2-week-old propagated plantlets were used. Leaf explants with a dimension ranging from 5 to 10 mm (Figure 1 b) were generated using a scalpel and subsequently cultured in a callogenesis induction medium containing 4,4 g L^{-1} of MS with vitamins, 30 g L^{-1} of sucrose, 1 mg L^{-1} of BAP, 3 mg L^{-1} of 1-naphtaleneacetic acid (NAA), 5 mg L^{-1} ascorbic acid , 5 mg L^{-1} citric acid and 7 mg L^{-1} of plant agar (Menin et al., 2013). Culturing was performed for 6 weeks at 24±1°C with 2 different conditions of light/dark period to obtain a differential callus morphology: (i) full darkness for 6 weeks (white calli) (Figure 10b, above); (ii) full darkness for 4 weeks followed by 2 weeks with a 16/8 hours light/dark period (green calli and brown calli) (Figure 10b, center and below). After 6 weeks, for each of the 3 callus morphologies, samples were generated by collecting ~1 cm calli explants immediately frozen in liquid nitrogen and stored at -80°C. In total, three samples/callus morphology were collected and used for the subsequent RNA extraction

procedure (see paragraph “RNA extraction”). Differently, for gene transfer experiments, SAMs were obtained by dissecting vegetative apexes from propagated virus-free clones. Isolated SAMs were maintained in the previously mentioned propagation medium for 3 days at growth chamber conditions and then used in transformation procedures (see paragraph “Plant genetic transformation procedures”).

The Romanesco C3 varietal type was also involved in this research for gene transfer experiments. The Vitroplant Italia S.r.l. (Cesena, FC, Italy) kindly provided i*n vitro* plantlets cultured in the previously mentioned propagation medium. Plants were propagated at normal growth chamber conditions for 2-3 weeks and then used for further plant transformation procedures as described later.

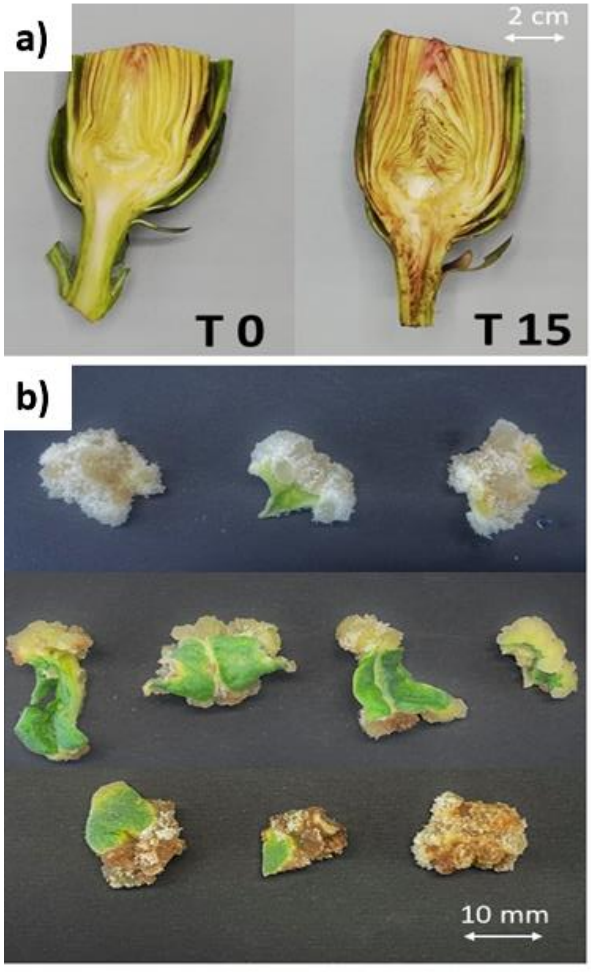


Figure 10. **Overview of globe artichoke tissue samples collected for gene expression analysis of *PPO* genes.** (a) Pictures showing capitulum sections of Violetto di Toscana variety at time zero (T0) and 15 minutes (T15) after cutting. (b) Pictures showing the three different callus morphologies in the Spinoso di Sardegna varieties, in order white, green and brown. Unit bars are reported.

2.2.2 RNA extraction

RNA was extracted from collected plant samples using the "Spectrum plant total RNA kit" (Sigma-Aldrich, St. Louis, USA), according to manufacturer's instructions. Extracted RNA was treated with DNase I (Thermo Fisher Scientific) to remove contaminant genomic DNA and quantified on the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific).

2.2.3 Synthesis of cDNA and RTqPCR analysis

For each sample, synthesis of cDNA was performed by reverse transcription using 1 µg of extracted RNA and the "High-Capacity cDNA Reverse Transcription Kit" (Applied Biosystems, USA), following protocol's instructions. The produced cDNA was used in RTqPCR analysis.

RTqPCR reactions were conducted on a "StepOnePlus Real-Time PCR system" (Applied Biosystem), in 96-well plates, in technical duplicates and three biological replicates, with 10 ng of starting cDNA, the "Power SYBR Green PCR Master Mix" (Applied Biosystem, USA) and the couples of primers reported in Table 1 and in Cerruti et al. (2019). The following PCR program was used: 1 cycle of 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Regarding the primers used in this analysis, two couples of primers/*PPO* were designed to specifically amplify each *PPO* and tested by a preliminary analysis based on the standard curve method. Thus, for each *PPO*, the couple of primers showing best parameters (slope around -3,5; R^2>0,98; eff% from 90 to 100) was selected and used in RTqPCR reactions (Table 1). The obtained RTqPCR data were quantified using the $2^{-\Delta\Delta Ct}$ method based on Ct values of *PPO* genes and *Actin* (*ACT*) (primers were retrieved from Cerruti et al., 2019) used as housekeeping gene. For data obtained from the Violetto di Toscana varietal type, TBI-C1-T0-R1 (Violetto di Toscana-internal bract-time 0-biological replicate 1) was used as internal reference. Differently, for data obtained from the Spinoso di Sardegna varietal type, Dark-R1 (Biological Replicate 1) was used as internal control. For statistical analysis of data, the IBM SPSS statistical software was used to perform a one-way ANOVA test followed by a Tukey's HSD (p-value ≤ 0.05) test to assess differences between each value (the latter corresponding to the mean of three biological replicates).

Table 1. **List of *PPO* primers used for gene expression analyses in RTqPCR.** Product size and Tm are respectively indicated as bp and °C.

Gene	Primer	Sequence (5'-3')	Product size	Tm
PPO1 (V2_02g003610.1.01)	P Fw	GATTTCCAAAACTCTGCCAG	186	62
	P Rev	AACACCAGACTCGATTGGTC		62
PPO2 (V2_02g003620.1.01)	P Fw	GTGCAATGCCACCGATAACA	161	59,19
	P Rev	CGATGTCAGGCGTGGTGATA		59.90
PPO3 (V2_02g003630.1.01)	P Fw	TTTCTTTTCCACCTCCCGCC	77	60,54
	P Rev	CGCAAGTATAATCGGCAGAGAC		59,27
PPO4 (V2_02g003640.1.01)	P Fw	GCAGGTAGTTTCTCGCAGGT	145	60,04
	P Rev	CGTCCCTTGGTACCAGTGTC		60,04
PPO5 (V2_02g003650.1.01)	P Fw	GGGTGGTCAAGCATGCATTC	98	59,83
	P Rev	TGGACGTAGCAGCTGTTTGT		59,89
PPO6 (V2_08g003420.1.01)	P Fw	TGCCACACAAACACAAGGGT	127	60,61
	P Rev	CCGGATCTTGGAACCACTGT		59.68
PPO7 (V2_12g010610.1.01)	P Fw	CAAACCATCGCCATGCAGTT	79	59,76
	P Rev	TTTCTCCGGTCAACCTTGCC		60,54
PPO8 (V2_17g002880.1.01)	P Fw	GCTTCTCCACTCTTCCCACC	113	60,04
	P Rev	TTCAGGGTGGTTGGTGTCAC		60,11
PPO9 (V2_17g002890.1.01)	P Fw	CTTCTTTTAGCTCGGCCGCC	59	61,71
	P Rev	GTTGGTCTTGTTGGTGGTGG		59,26
PPO10 (V2_17g002900.1.01)	P Fw	GGCTTCTCTTCCTTCTCCGG	135	59,82
	P Rev	CGATGGGTTTGCTTTGCTGA		59,40
PPO11 (V2_ScYrq3g_1694g000100.1.01)	P Fw	CCACATAAGCAGAGGGACGG	103	60,18
	P Rev	CATCATCATCACCCACCTCCA		59,51

2.3 Agrobacterium-mediated plant genetic transformation

2.3.1 Construction of plant transformation vectors

In total, for transformation of Spinoso di Sardegna and Romanesco C3 varietal types, 4 different transformation vectors were involved: (i) the pK7WGF2-GFP (ii) the pGUS-INT, (iii) the pTRANS221-6 (*WUS* and *BBM*) and (iv) the pDGB_α2_CcPDS.

The pK7WGF2-GFP (Karimi et al., 2002) vector was already available in the laboratory. It contained a transformation cassette carrying the *NPTII* and *GFP* genes under the control of a *Nopaline synthase* (*NOS*) promoter and a *Cauliflower Mosaic Virus 35S* (*CaMV35S*) promoter, respectively.

The pGUS-INT vector was kindly provided by the Istituto Per La Protezione Sostenibile Delle Piante (IPSP, Torino, Italy) and carried, between the left and right borders (LB and RB), the *NPTII* and *GUS* genes under the control of a *NOS* promoter and a *CaMV35S* promoter, respectively.

The pTRANS221-6 vector was also already available in the laboratory. It was previously assembled, according to the modular assembly procedure described by Čermák and colleagues (2017), to carry the *NPTII* gene regulated by the *CaMV35S* promoter and the *WUS* and *BBM* genes under the control of a *NOS* promoter and a *Zea mays Ubitquitin-1* (*ZmUbi1*) promoter, respectively (Maher et al., 2020).

The pDGB_α2_CcPDS was designed and assembled during the activities of this thesis and it was programmed to specifically target, *via* a CRISPR/Cas9 system, the *PDS* visual marker gene. At first, to identify all *PDS* genes in *C. cardunculus* var. *scolymus*, the PDS amino acidic sequence previously annotated in the v.1.0 proteome (Scaglione et al., 2016) and available in the Globe Artichoke Genome Database was used for a BLASTp analysis against the v.2.0 globe artichoke reference proteome (Acquadro et al., 2020), using an e-value threshold of $1e^{-5}$. One PDS protein was identified, thus its corresponding genomic sequence (V2_03g022490.1.01) isolated and used for gRNA designing. Two gRNAs were chosen with CRISPOR software (http://crispor.tefor.net/) using the *PDS* gene as target sequence, the v.2.0 globe artichoke genome as reference and a 20bp-NGG (-SpCas9) as PAM. The obtained gRNAs were filtered based on: (i) guanidine as first base (due to the use of U6 promoter); (ii) the ability to target in exon sequences. Among the remaining gRNAs, two gRNAs, showing best parameters according to specificity, efficiency, outcome and OT scores, were selected (Table 2). Thus, the GoldenBraid 2.0 version (GB 2.0) was used as Modular DNA Construction method, which permits the assembly of a construct with transcriptional units made of prefabricated modules (GBparts) (*GOLDENBRAID 2.0*, 2015). A multipartite assembly reaction and two binary assemblies were set up to create the pDGB_ α2_Cc_PDS. At first, by a restriction-ligase reaction, gRNA1 and gRNA2 were respectively inserted into pDGB_α1 and pDGB_α2 plasmids

between the *A. thaliana Ubiquitin-6* (*AtU6*) promoter and the scaffold sequence. Next, resulting plasmids were assembled in the same destination vector (pDGB_Ω2). The obtained pDGB_Ω2 vector was assembled with a pDGB_Ω1 vector containing *Cas9* and *NPTII* sequences, producing the final transformation vector pDGB_ α2_Cc_PDS. During the cloning procedures required by the GB 2.0 protocol, different primers were used to screen the right assembly of each plasmid assembled (Table 3). Moreover, the assembly of the final pDGB_α2_CcPDS vector was confirmed by Sanger sequencing (Sanger et al., 1997) using the sequencing service of the BMR Genomics S.r.l. (Padova, Italy). Sequencing results (fastq files) were checked using Benchling platform (https://www.benchling.com/) by sequence comparison alignment with an *in silico* pre-assembled pDGB_α2_CcPDS sequence containing all the GBparts. In detail, the T-DNA cassette of the produced pDGB_α2_CcPDS vector included: the *NPTII* gene regulated by *NOS* promoter and terminator; the *Cas9* gene cloned between a *CaMV35S* promoter and a *Nopaline Synthase terminator* (*TNOS*); gRNA1 and gRNA2 located between the *At6* promoter and the Scaffold sequence (see Figure 12 in the section 3.1 of cap. 3). Differently, the backbone region included: a Kanamycin resistance gene; a *Pseudomonas* plasmid pSV1 Replication region (pVS1-Rep\ori); a plasmid pBR322 Origin of Replication (pBR322\ori) (See Figure 12 in the section 3.1 of cap. 3).

Table 2. **List of gRNAs, targeting the *PDS* gene, used for the assembly of the pDGB_α2_CcPDS.** MIT specificity score (MIT); Cutting Frequency Degree Specificity Score (CFD); Doench '16 model (D); Mor.-Mateos model (MM); Out-of-Frame (OF); Indel (I); Off-target (OT).

				Efficiency		Outcome		
	gRNA + (PAM) Sequence	**MIT**	**CFD**	**D**	**MM**	**OF**	**I**	**OT**
gRNA 1	GGAGAAGAGAGGATCTGGAT (TGG)	91	95	48	44	73	78	50
gRNA 2	GGTCACCGATTGCAATTCCC (AGG)	99	99	56	33	63	77	5

Table 3. **List of primers used during GoldenBraid 2.0 version (GB 2.0) protocol for the assembly of the pDGB_α2_CcPDS.**

Primer ID	**Sequence (5'-3')**
Screening_α/Ω_for	CTTAGTTGCCGTTCTTCCGA
Atu6_for	AGAAGAGAAGCAGGCCCATT
Screen_for	CCCCAAGAAGAAGAGGAAGG
Screen_rev	AAACCTTTTCACGCCCTTTT
Scaffold_rev	CGACTCGGTGCCACTTTT

2.3.2 Electroporation of *A. tumefaciens* with plant transformation vectors

All the transformation plasmids were transferred into *A. tumefaciens,* GV3101 strain, by electroporation. For each transformation, around 70-100 ng of plasmid DNA were added to 40 µL of GV3101 electrocompetent cells and the resulting mixture was pipetted into a pre-chilled cuvette. The cuvette was thus placed into an "ECM 399 Exponential Decay Wave Electroporation System" and subjected to a 2,500-volt shock. Immediately, 800 µL of SOC medium (Sun et al., 2009) were added to the cuvette and the resulting mixture was collected and transferred into a microcentrifuge tube. The bacteria culture was incubated at 28 °C for 3 hours, with a 200-rpm shaking. After incubation, 100 µL of culture were plated on solid LB medium added with KAN (50 µg mL^{-1}) (pGUS-INT, pTRANS221-6 and pDGB_α2_CcPDS) or SPC (50 µg mL^{-1}) (pK7WGF2-GFP), and Rifampicin (RIF; 50 µg mL^{-1}). Plates were incubated for 3 days at 28°C to allow bacteria growth. Thus, one colony/plasmid was selected, grown overnight in liquid LB medium supplemented with the appropriate antibiotics and then stored as a glycerol stock at -80°C.

2.3.3 Plant genetic transformation procedures

Activation of A. tumefaciens

Before starting plant transformation protocols, the previously transformed *A. tumefaciens* was cultured and activated. The day before transformation, a 10 µL aliquot of the glycerol stock was pipetted into 20 mL of liquid LB medium supplemented with appropriate antibiotics and grown overnight at 28°C with a 200-rpm shaking. The following day, the optical density (OD) of the bacteria was calculated with a spectrophotometer (Beckman Coulter DU 730, Life Science UV/Vis Spectrophotometer). An OD of 0.5 was required for transformation. Thus, bacteria were centrifuged, pelleted and resuspended to 0.5 OD using liquid MS medium supplemented with 200 µM acetosyringone (AS) to induce the virulence of *A. tumefaciens.* The obtained mixture was incubated 4 hours at 80 rpm before to be used for transformation procedures as reported below.

Transformation of shoot apical meristems in the Spinoso di Sardegna varietal type

In the Spinoso di Sardegna varietal type, SAMs, obtained and maintained *in vitro* as described in the previous paragraph "Plant material, growth condition and sampling", were involved for transformations. Two stocks of *A. tumefaciens* previously transformed with respectively the pK7WGF2-GFP and the pGUS-INT vectors were used taking advantage of two different transformation methods: microinjection and co-culture.

Microinjection was performed according to the procedure described by Baskaran et al. (2006): by using a sterile syringe (0.1 mm needle), approximatively 2 µL of activated *A. tumefaciens* were injected 3 times into SAM region (Figure 11a). Differently, co-culture was performed by dipping SAMs directly into the *A. tumefaciens* solution (Figure 11b). Dipped

SAMs were incubated for 20 min at 150 rpm. In both cases, microinjected and co-cultured SAMs were blotted on a filter paper and cultured on the previously mentioned propagation medium supplemented with 200 µM AS for 3 days in the dark. Thus, SAMs were washed 3 times with sterile water and transferred to a new propagation medium added with timentin (TIM) (150 µg mL^{-1}). SAMs were thus maintained at normal growth chamber conditions and medium was refreshed every 4 weeks.

Transformation of leaf explants in the Romanesco C3 varietal type

In the Romanesco C3 varietal type, 2-weeks-old plantlets, maintained *in vitro* as described in the previous paragraph "Plant material, growth condition and sampling", were involved for transformations. Two stocks of *A.tumefaciens* previously transformed with respectively the pTRANS221-6 and the pDGB_α2_CcPDS vectors were used, separately and in combination (1:1 v/v), to transform leaf explants using a co-culture method (Figure 11c). Leaves of *in vitro* plantlets were collected and cut by a scalpel to generate leaf explants of approximatively 0.5 cm in size. Thus, explants were immersed into the *A. tumefaciens* solution and incubated for 20 min at 150 rpm. Subsequently, explants were blotted on a filter paper and cultured on the previously mentioned callogenesis induction medium (see paragraph "Plant material, growth condition and sampling") (Menin et al., 2013) supplemented with 200 µM AS for 3 days in the dark. Following co-culture, explants were washed 3 times with sterile water and transferred to the same callogenesis induction medium added with KAN (50 µg mL^{-1}) and timentin (TIM) (150 µg mL^{-1}). Transformed leaf explants were maintained at normal growth chamber conditions and medium was refreshed every 4 weeks.

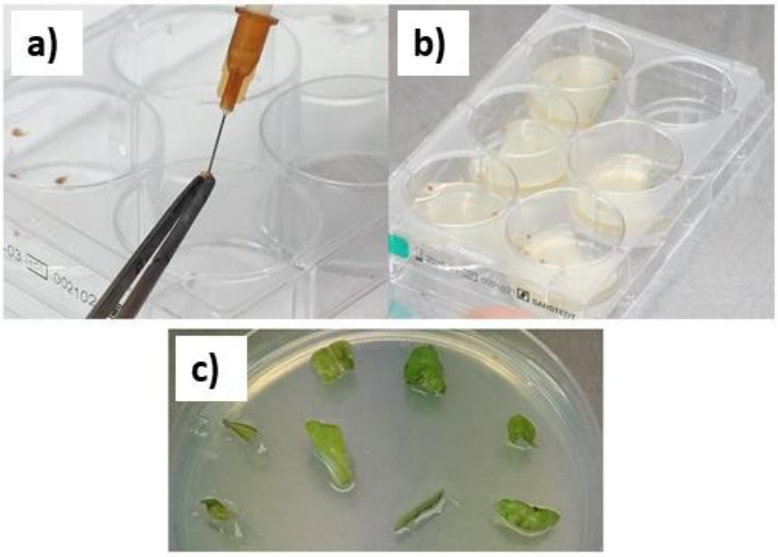


Figure 11. **Pictures showing steps of different plant transformation procedures used in this research.** (a) Microinjection of SAMs in the Spinoso di Sardegna varietal type. (b) Co-culture of SAMs in the Spinoso di Sardegna varietal type. (c) Co-culture of leaf explants in the Romanesco C3 varietal type.

3. RESULTS

3.1 Identification, structure and phylogenetic analysis of globe artichoke *PPO* genes

Using a BLASTp analysis with the previously annotated PPOs (Scaglione et al., 2016), the presence of 11 *PPO* genes was confirmed in the new globe artichoke reference genome (v.2.0) (Acquadro et al., 2020). Genomic and protein features of the identified *PPOs* are summarized in Table 4.

Table 4. **Overview of genomic and protein features of *PPO* genes identified in the v.2.0 globe artichoke reference genome.** Chromosome (Chr); Open reading frame (ORF); amino acid (aa).

Locus	Gene name	Chr	Chr location	Strand	Length	Exon/Intron	ORF (bp)	aa	Protein domain	Pfam domain
V2_02g003610.1.01	PPO1	2	3791255-3792244	+	989	Ex: 0-989	989	329	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_02g003620.1.01	PPO2	2	3811850-3813700	-	1850	Ex: 0-1850	1850	616	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_02g003630.1.01	PPO3	2	3822577-3823984	+	1407	Ex1: 0-136 Int1: 137-441 Ex2: 442-1407	1104	367	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_02g003640.1.01	PPO4	2	3831196-3844009	-	12813	Ex1: 0-1024 Int1: 1024-10967 Ex2: 10968-12813	2871	956	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_02g003650.1.01	PPO5	2	3860408-3862336	+	1928	Ex1: 0-446 Int1: 447-770 Ex2: 771-1928	1605	534	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_08g003420.1.01	PPO6	8	3768876-3769760	+	884	Ex: 0-884	884	294	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_12g010610.1.01	PPO7	12	16811702-16813483	-	1781	Ex: 0-1781	1781	593	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_17g002880.1.01	PPO8	17	3179409-3181226	+	1817	Ex: 0-1817	1817	605	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_17g002890.1.0	PPO9	17	3195388-3206783	+	11395	Ex1: 0-1811 Int1: 1812-10615 Ex2: 10616-11395	2532	863	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_17g002900.1.01	PPO10	17	3212811-3214601	-	1790	Ex: 0-1790	1790	596	Tyrosinase PPO1_DWL PPO1_KFDV	Pfam 00372 Pfam 00264 Pfam 00264
V2_ScYrq3g_1694g000100.1.01	PPO11	ScYrq3g_1694	2494-2931	+	437	Ex: 0-437	437	145	PPO1_KFDV	Pfam 00264

Overall, *PPO* genes were found to be localized on different chromosomes and on different strands. In detail, *PPO1-5* mapped on chromosome 2, *PPO6* mapped on chromosome 8, while *PPO7* and *PPO8-10* mapped on chromosomes 12 and 17 respectively. Differently, *PPO11* localized on the ScYrq3g_1694 scaffold, thus its exact position in the genome is still unknown (Table 4). *PPO* gene sequences were highly variable in length, ranging from 437 bp (of *PPO11*) to 12813 bp (of *PPO4*). Only *PPO3*, *PPO4*, *PPO5* and *PPO9* showed the presence of one intron in the sequence (Table 4). The alignment of all the *PPO* CDSs, made with ClustalW Multiple Alignment, highlighted a high nucleotide similarity between *PPO1* and *PPO2* (98.3), *PPO2* and *PPO3* (97.5), and *PPO1* and *PPO3* (96.0) (data not shown).

PPO proteins ranged in size from 145 (of *PPO11*) to 956 (of *PPO4*) amino acids (aa) and were characterized by the same protein functional domains: a common central domain of tyrosinase, a polyphenol oxidase middle domain (PPO1_DWL) and a domain of unknown function (DUF_B2219) containing the KFDV conserved motif (Figure 14). Interestingly, *PPO11* showed only the PPO-KFDV domain.

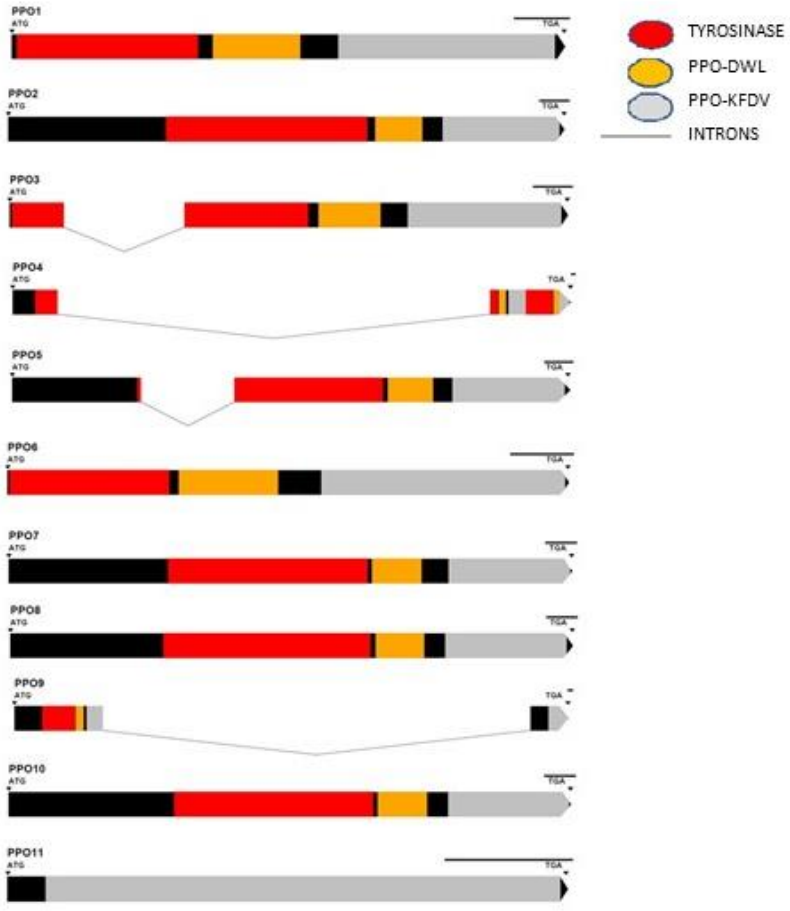


Figure 12. Schematic representation of *PPO* genes. The *PPO* genomic sequences are illustrated including exon (boxes) and intron (lines) regions, and the corresponding protein domains codified.

In order to better characterize globe artichoke *PPO*s, a phylogenetic analysis was performed to acquire information on their structural and functional conservation. The phylogenetic tree was generated by using the 11 isolated globe artichoke PPOs and a set of PPOs retrieved from ten species of *Asteraceae* (*Taraxacum officinale, Tanacetum cinerariifolium, Lactuca sativa, Heliantus annuus, Gerbera jamesonii, Cynara cardunculus* subsp. *Cardunculus, Dahlia pinnata, Mikania micrantha, Lactuca saligna, Erigeron canadensis*) and three of *Solanaceae* (*Solanum tuberosum, Solanum melongena, Solanum lycopersicum*) (Figure 13).

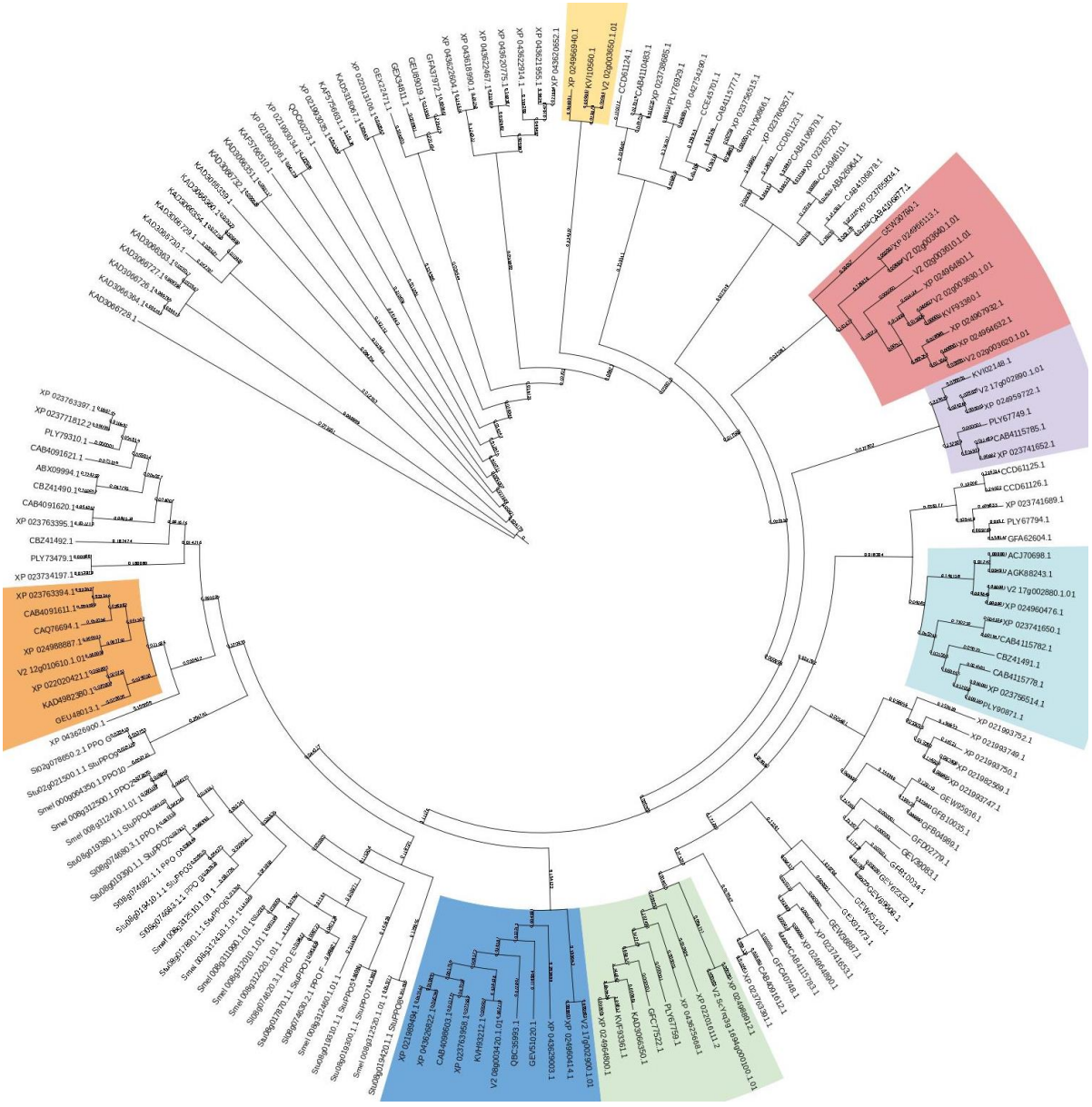


Figure 13. Phylogenetic tree of PPOs. Complete representation of the phylogenetic tree comprehensive of PPOs from globe artichoke and other 13 species (10 *Asteraceae* and 3 *Solanaceae* species). Clusters including globe artichoke PPOs are highlighted with different colors.

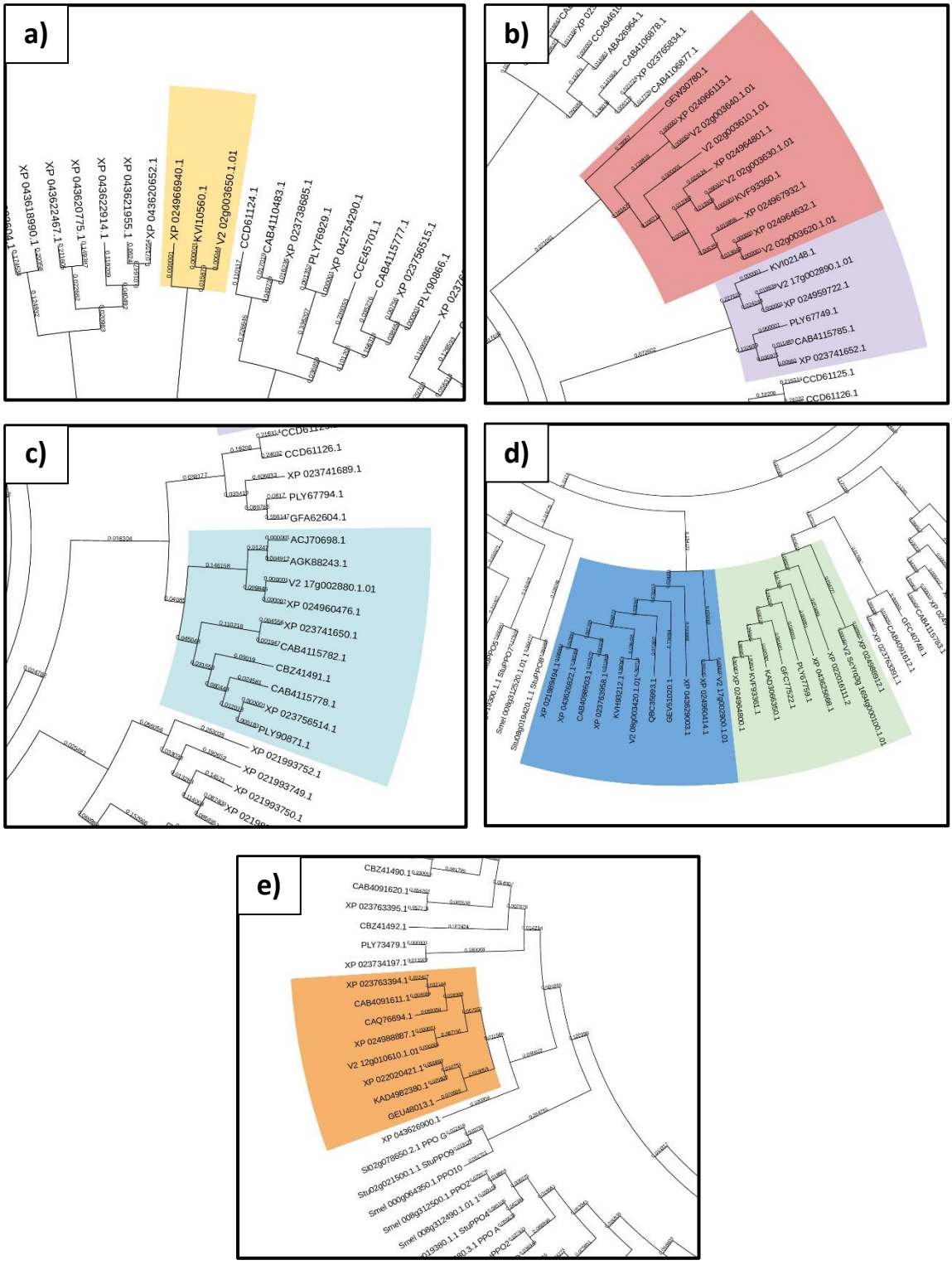


Figure 14. Focus on specific clusters of the phylogenetic tree containing globe artichoke PPOs. (a) PPO5. (b) PPO1-4 (red) and PPO9 (violet). (c) PPO8. (d) PPO6 and PPO11. (e) PPO7.

Overall, a strong sequence similarity was observed for PPOs of globe artichoke (*C. cardunculus* var. *scolymus*) and *C. cardunculus* subsp*. cardunculus* (wild cardoon), due to the evolutive proximity of these species. In detail, PPO5 clustered together with two PPOs from *C. cardunculus subsp. cardunculus* (Figure 14a). PPO1-4 clustered with two homologs from *C. cardunculus* subsp. *cardunculus* and *T. cinerariifolium* (Figure 14b). PPO8 (Figure 14c) and PPO9 (Figure 14b) generated two different clusters, each one containing different PPOs from *L. sativa* and *L. saligna*. In literature, these PPOs were functionally characterized and found to be activated by different physical stimuli, such as pH, heat and light (Doğan & Salman, 2007). PPO8 was also grouped with PPO from *T. officinalis* (Figure 14c); this species has been studied for its high content of PPOs in latex and its role in wound sealing (Wahler et al., 2009). PPO6 and PPO10 clustered with PPOs belonging to *E. canadensis*, *T. cinerariifolium*, *G. jamesonii*, *C. cardunculus* subsp *cardunculus*, *L. sativa*, *L. saligna* and *H. anuus*. PPO11 grouped with PPOs from C. *cardunculus* subsp*. cardunculus, H. annuus, E. canadensis, L. sativa, T. cinerariifolium and M. micrantha* (Figure 14d). Finally, PPO7 clustered with different PPOs from *T. officinalis*, *L. saligna* and *L. sativa* (Figure 14e).

3.2 Genetic variations in the *PPO* genes across four agronomically important globe artichoke varietal types

The presence of allelic variants was evaluated in the *PPO* genomic sequences of four agronomically and economically important globe artichoke varietal types: Violetto di Toscana (VT), Violetto di Sicilia (VS), Romanesco C3 (C3) and Spinoso di Palermo (SP) (VT in Figure 15 and VS, C3 and SP in Figure 16). Overall, SNPs/Indels profile was similar among the four varietal types, showing a range of 600-700 polymorphisms/varietal type (Figure 15 and 16). Across the 4 varietal types, *PPO1* and *PPO11* showed the minor number of variants (on average 6 and 7 respectively) while *PPO4* and *PPO9* showed the major number of variants (on average 287 and 155 respectively) (Figure 15 and 16, left). Regarding their localization in exon and intron sequences, for *PPO4* and *PPO9* the majority of variants occurred in intron regions while for *PPO3* and *PPO5* in exon regions (Figure 15 and 16, left). The type of variants was also highly conserved among the four varietal types: only a 2±0,5% was insertions and deletions (indels), while the vast majority was represented by SNPs (substitutions) (Figure 15 and 16, right).

Each variant identified was thus evaluated for its impact on the corresponding gene translation. In VT five high impact alterations were found in three genomic sequences: two in *PPO4* (1 bp substitutions), one in *PPO5* (4 bp insertion) and two in *PPO9* (20 bp deletion and 1 bp substitution) (Table 5). In VS and in SP the same five Indels were identified in exon sequences of *PPO2* (6 bp deletion), *PPO5* (4 bp insertion), *PPO8* (6 bp deletion) and *PPO9* (15 bp insertion and 20 bp deletion) (Table 5). In C3 six Indels were in total observed in *PPO2* (8 bp deletion), *PPO4* (1 bp deletion), *PPO5* (4 bp insertion), *PPO8* (6 bp deletion), PPO9 (15 bp insertion) and *PPO10* (3 bp insertion). Remarkably, the 4 bp insertion in VT *PPO5* and the 6 bp deletion in VS, SP and C3 *PPO8* were found to be in homozygosis status and located at the beginning of the gene sequences, thus potentially leading to a significative impact on the correct gene translation.

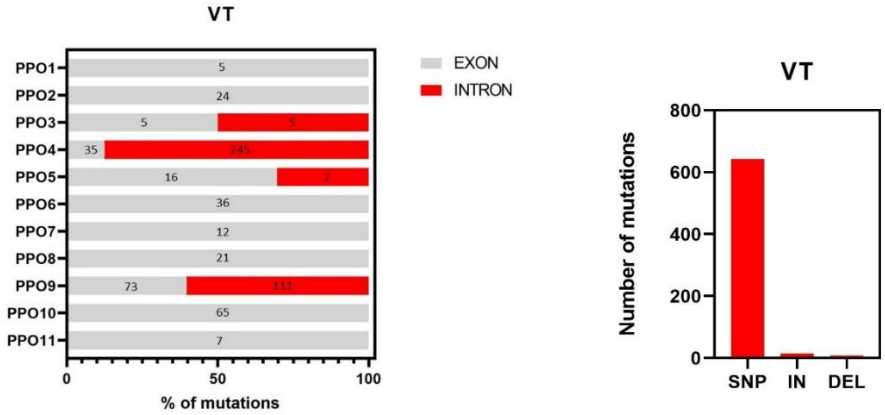


Figure 15. SNPs/indels analysis in *PPO* genes of Violetto di Toscana varietal type. On the left, the number of variants per *PPO*, divided in exon and intron regions, is reported. On the right, the different types of variants (substitutions, insertions and deletions) are indicated. Data indicate Violetto di Toscana (VT)

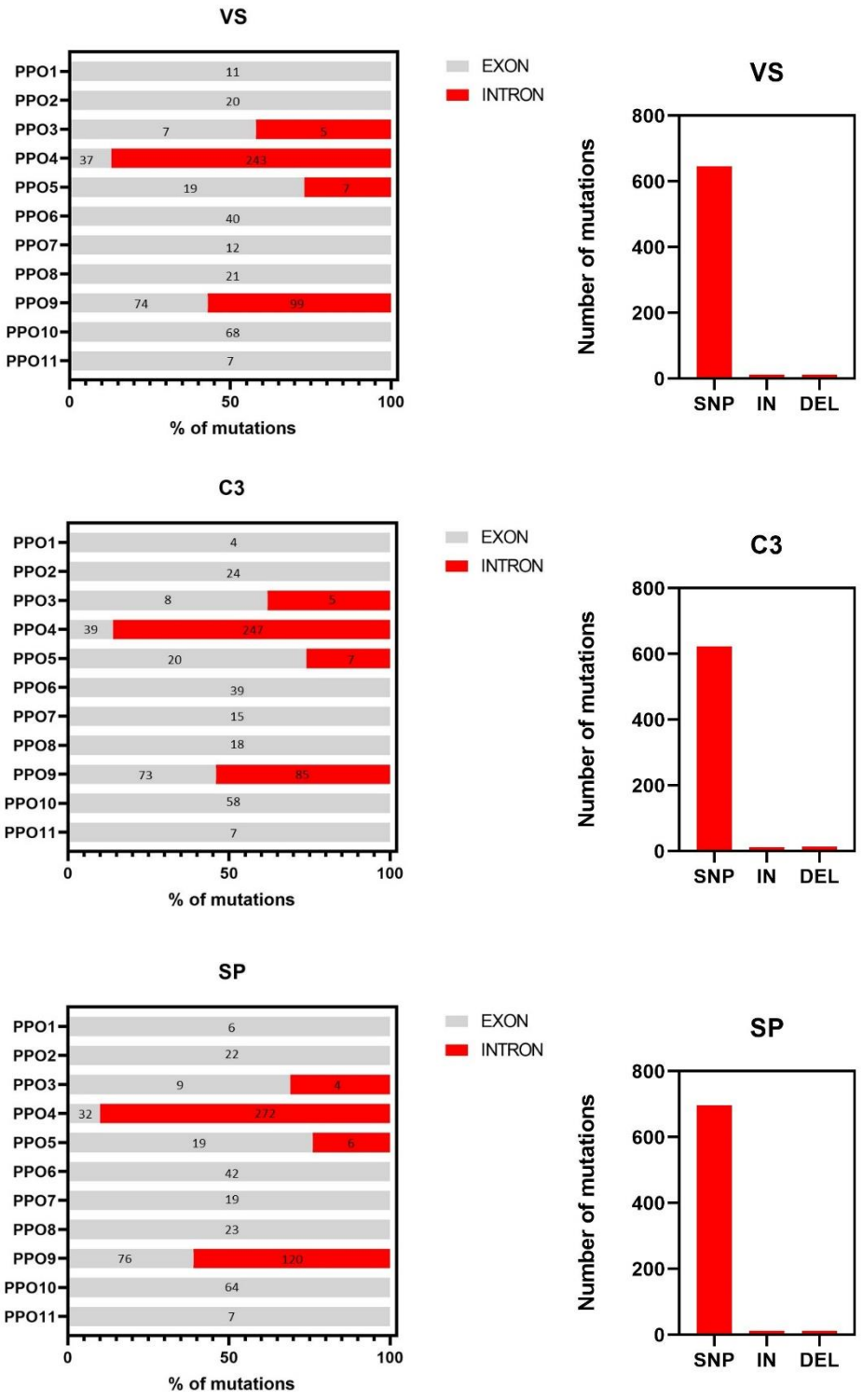


Figure 16. SNPs/indels analysis in *PPO* genes of Violetto di Sicilia, Romanesco C3 and Spinoso di Parlermo varietal types. On the left, the number of variants per *PPO*, divided in exon and intron regions, is reported. On the right, the different types of variants (substitutions, insertions and deletions) are indicated. Data indicate Violetto di Sicilia (VS, above), Romanesco C3 (C3, in the middle) and Spinoso di Parlermo (SP, below).

Table 5. Summary of high impact variants in the *PPOs* of four globe artichoke varietal types. For each variant, the position into the genome, and the reference and alternative sequences are reported.

Varietal type	Gene	Gene position	SNP/Indel Position	Reference	Alternative
VT	V2_02g003640.1.01	3831196 - 3844009	3831685	G	C
	V2_02g003640.1.01	3831196 - 3844009	3842888	G	C
	V2_02g003650.1.01	3860408 - 3862336	3860433	AAC	AACGTAC
	V2_17g002890.1.01	3195388 - 3206783	3206527	TTGTCAATGACAAAGACAATGT	TT
	V2_17g002890.1.01	3195388 - 3206783	3206699	C	A
VS	V2_02g003620.1.01	3811850 - 3813700	3812906	TGAGTTGGAGTTGG	TGAGTTGG
	V2_02g003650.1.01	3860408 - 3862336	3860433	AAC	AACGTAC
	V2_17g002880.1.01	3179409 - 3181226	3180165	GATACTA	G
	V2_17g002890.1.01	3195388 - 3206783	3206220	CCC	CCCGGTGCTGCTCTGTCC
	V2_17g002890.1.01	3195388 - 3206783	3206527	TTGTCAATGACAAAGACAATGT	TT
C3	V2_02g003620.1.01	3811850 - 3813700	3812906	TGAGTTGGAGTTGG	TGAGTTGG
	V2_02g003640.1.01	3831196 - 3844009	3843437	ACC	AC
	V2_02g003650.1.01	3860408 - 3862336	3860433	AAC	AACGTAC
	V2_17g002880.1.01	3179409 - 3181226	3180165	GATACTA	G
	V2_17g002890.1.01	3195388 - 3206783	3206220	CCC	CCCGGTGCTGCTCTGTCC
	V2_17g002900.1.01	3212811 - 3214601	3214566	AGCGGCGGCGGCG	AGCGGCGGCG
SP	V2_02g003620.1.01	3811850 - 3813700	3812906	TGAGTTGGAGTTGG	TGAGTTGG
	V2_02g003650.1.01	3860408 - 3862336	3860433	ACC	AACGTAC
	V2_17g002880.1.01	3179409 - 3181226	3180165	GATACTA	G
	V2_17g002890.1.01	3195388 - 3206783	3206220	CCC	CCCGGTGCTGCTCTGTCC
	V2_17g002890.1.01	3195388 - 3206783	3206527	TTGTCAATGACAAAGACAATGT	TT

3.3 *In silico* prediction of a putative TFBS based-gene regulatory and functional profile of globe artichoke *PPO* genes

In order to identify putative TFBSs and corresponding TFs involved in the regulation of *PPOs* expression, the promoter sequence of the 11 *PPOs* (1Kb upstream of the ATG translation start site) was screened by the PlantPAN software, using the *A. thaliana* TFBS database as reference. The identified TFBSs were distributed fairly evenly along the *PPO* promoters and ranged from 46 (*PPO2*) to 150 (*PPO10*) with an average of 68 TFBSs/gene promoter (Figure 17, left). The corresponding TFs able to recognize the identified TFBSs grouped into a multitude of TF families (Figure 17, left). Interestingly, the number of TF families was not proportional to the number of corresponding TFBSs. For instance, *PPO10* promoter (showing the highest number of TFBSs) was associated to 26 TF families while *PPO1* promoter (showing an average number of TFBSs) was linked to 29 TF families (Figure 17, left).

Using the available gene ontology information (retrieved by the PlantTFDB database) concerning the biological processes associated with each TF identified, a putative regulatory and functional profile for each *PPO* promoter was generated (Figure 17, right). In detail, *PPO* promoters were found to be potentially regulated during several biological processes divided into three different macro-groups: (i) hormone biogenesis and signaling pathways; (ii) growth and development; (iii) physiological stimulus and stress response.

With regard to the first group, interestingly the activity of *PPO* promoters (mainly *PPO4*, *PPO7*, *PPO9* and *PPO10*) was observed to be associated with ethylene, salicylic acid and jasmonic acid pathways, that are well known to be involved in the response to different abiotic and biotic stresses.

Moreover, concerning the second group, all the *PPO* promoters appeared to be potentially regulated (*PPO5*, *PPO7* and *PPO10* with high impact) during the inflorescence growth and development. *PPO7* and *PPO10* promoters showed a potential activity also during senescence, which is in general associated to tissue browning.

Finally, with regard to the third group, a potential regulation of *PPO4*, *PPO7* and *PPO10* promoters resulted associated with plant response to biotic stresses, such as bacteria and fungi. Notably, all *PPO* promoters showed a high impact regulation upon light stimuli.

Prom	TFBSs	Families
PPO1	67	29
PPO2	46	21
PPO3	58	24
PPO4	68	23
PPO5	62	21
PPO6	48	18
PPO7	92	23
PPO8	58	19
PPO9	52	22
PPO10	150	26
PPO11	51	23

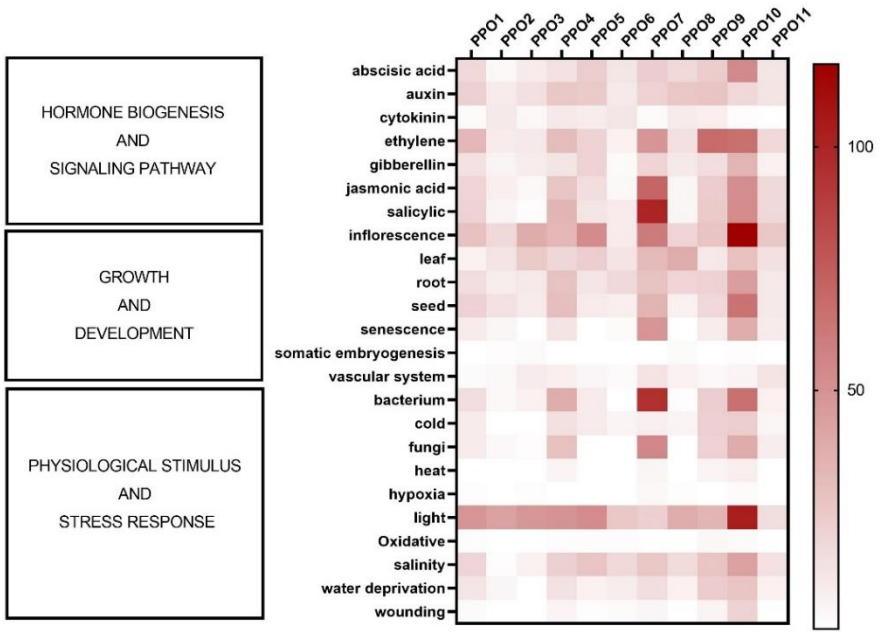


Figure 17. Putative TFBSs-based regulatory and functionally profile of globe artichoke *PPOs.* On the left, the number of TFBSs and corresponding TF families are reported for each *PPO* promoter. On the right, a heat map showing a putative TFBS-based functional profile of *PPO* promoters is illustrated.

3.4 Tissue- and organ-specific expression pattern of *PPO* genes in globe artichoke

Besides *in silico* analysis, the examination of putative changes in the expression profile of *PPO* genes in different plant tissues and in response to environmental stimuli, such as wounding, may provide insights into the biological roles of this important globe artichoke gene family. Thus, the expression pattern of *PPO* genes was investigated by RTqPCR analyses in the VT globe artichoke varietal types (selected for its high browning effect) in different tissues (i.e. capitulum, leaf and stem) and upon wounding (T0, i.e. basal expression; T15, i.e. expression 15 minutes after cutting).

When *PPOs* basal expression level was estimated an eterogeneous expression profile was observed (Figure 18). A clear tissue-specificity was identified for *PPO7*, which was expressed only in TFC1 and TFC2. *PPO8* was mainly expressed in TBEC1 and TSC1. Also *PPO10* resulted higlhy specific, being expressed particularly in TFC2. *PPO3* was expressed at higher level in leaves (TFC1 and TFC2) but also in the capitulum and in the stem. *PPO1, PPO2* and *PPO6* were upregulated in secondary capitulum (TBIC2 and TRC2), as well as *PPO11* (TRC2 and TFC2). Interestingly, *PPO9* was not detected in all the analysed tissues.

Differently, 15 minutes after cutting, receptacle, internal and esternal bracts of primary and secondary capitulum were analyzed (Figure 19). *PPO1, PPO2, PPO6* and *PPO11* showed significant increasing of expression levels, especially in secondary capitulum (TBIC2). Even *PPO10*'s expression is upregulated after 15 min in C2 tissues: TBEC2, TBIC2 and TRC2. Instead, in *PPO3*, *PPO4*, *PPO5*, *PPO7* and *PPO8* no differences were observed. *PPO9* wasn't detected either at T15.

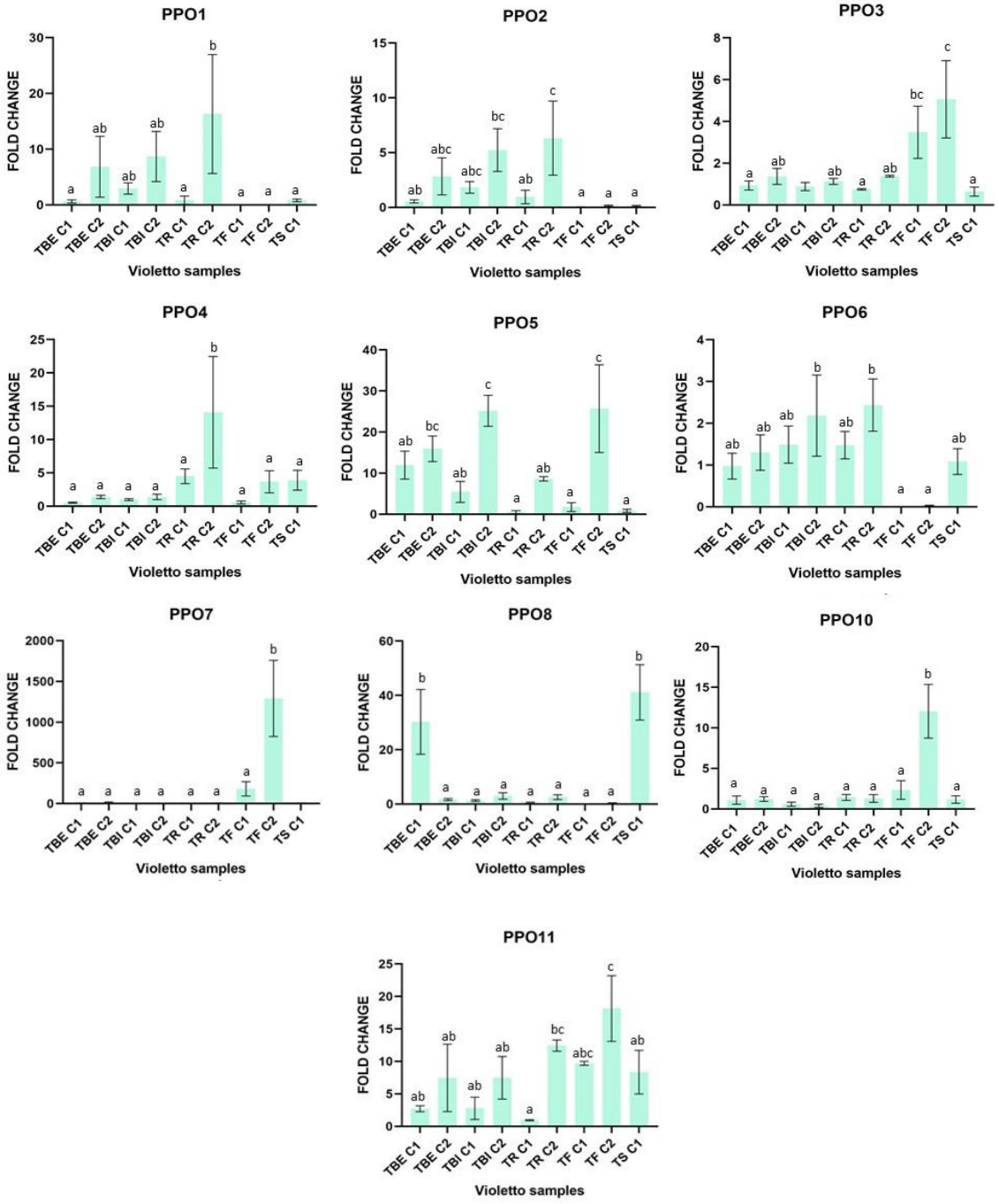


Figure 18. Expression level of VT *PPO* genes in different plant tissue. Each graph shows the basal expression level (Fold change) of one *PPO* in the tested tissues. Bars indicate mean values ± SE of three biological replicates. Letters indicate statistically significance differences of datasets according to a one-way ANOVA test followed by a Tukey's HSD (p-value ≤ 0.05). Violetto di Toscana (T); Bract (B); External (E); Internal (I); Primary Capitulum (C1); Secondary Capitulum (C2); Receptacle (R); Leaf (F); Stem (S).

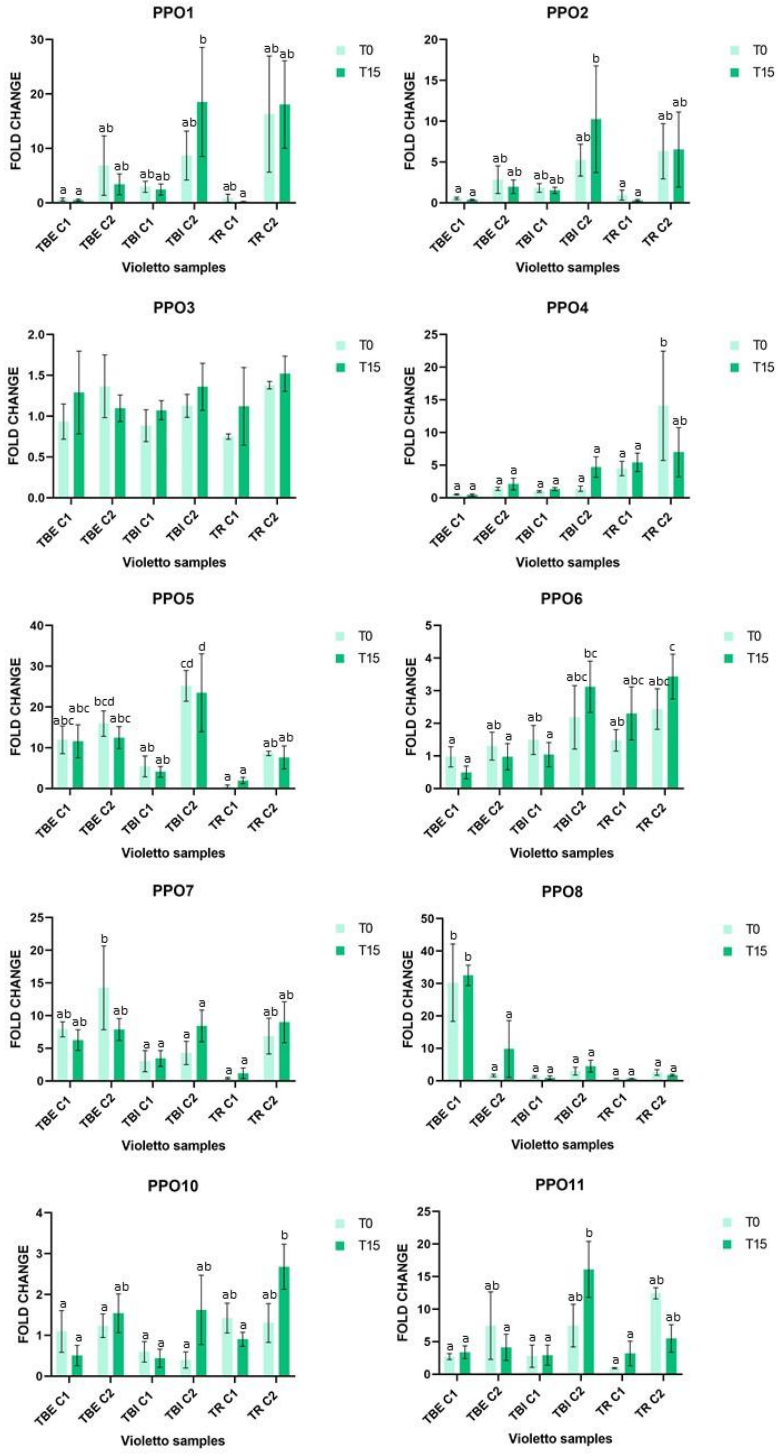


Figure 19. Expression level of VT *PPO* genes upon wounding. Each graph shows the expression level (Fold change) of one *PPO* in the tested tissues at T0 and T15 after cut. Bars indicate mean values ± SE of three biological replicates. Letters indicate statistically significance differences of datasets according to a one-way ANOVA test followed by a Tukey's HSD (p-value ≤ 0.05). Violetto di Toscana (T); Bract (B); External (E); Internal (I); Primary Capitulum (C1); Secondary Capitulum (C2); Receptacle (R).

Moreover, in order to acquire more information regarding the involvement of *PPOs* in browning during *in vitro* culture, expression levels of *PPO*s were also evaluated in different callus morphologies of Spinoso di Sardegna varietal types (selected for the availability of efficient *in vitro* maintenance protocols) (Figure 20). Three types of calli were selected: white, green and brown. Our data indicated that *PPO5, PPO6, PPO7* and *PPO11* were significantly up-regulated in brown calli compared to green and white calli. Differently, *PPO3* and *PPO8* showed a significant activation in white calli. *PPO10* was significantly expressed in green calli. Finally, *PPO1*, *PPO2*, *PPO4* and *PPO9* resulted low expressed in all types of calli.

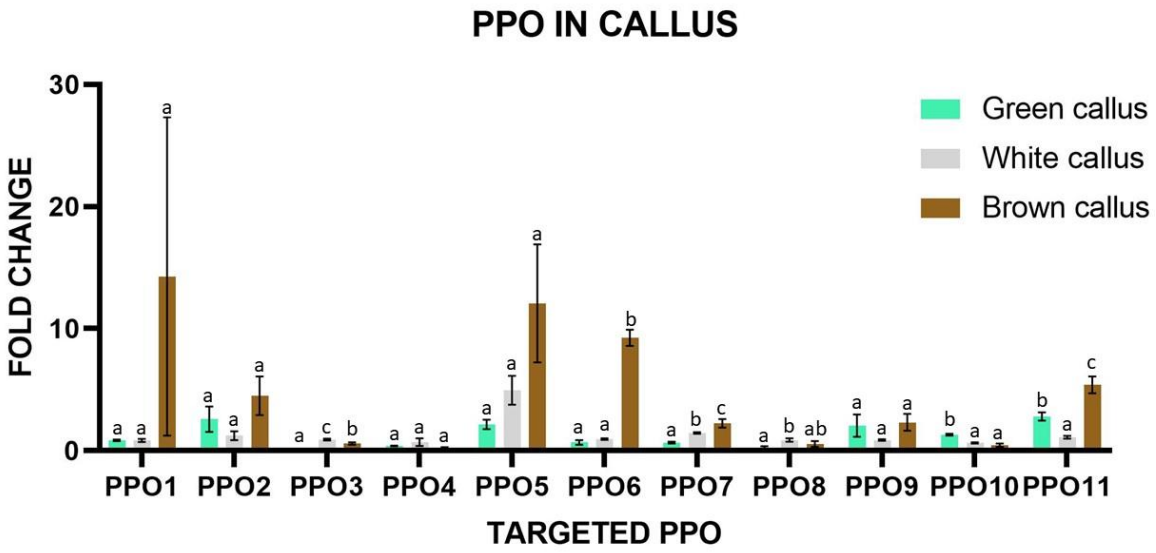


Figure 20. Expression levels of *PPO*s in different callus morphologies of Spinoso di Sardegna varietal type. Three different callus phenotypes were tested in RTqPCR analysis for all the *PPO*s. Bars indicate mean values ± SE of three biological replicates. Letters indicate statistically significance differences of datasets according to a one-way ANOVA test followed by a Tukey's HSD (p-value ≤ 0.05).

3.5 Construction of transformation vector with GoldenBraid protocol

Using CRISPOR software two gRNAs targeting the *PDS* gene (on exon 1 and exon 3) were designed and domesticated, adding prefix and suffix to make them suitable for GB cloning (Figure 21).

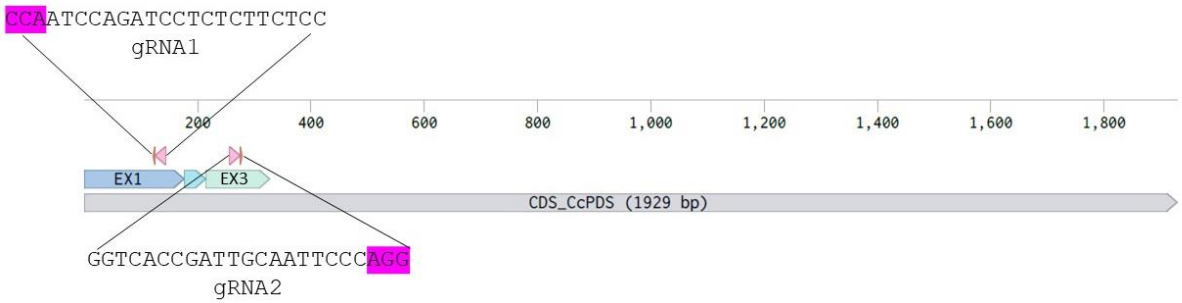
GoldenBraid 2.0 protocol has been used to assemble the CRISPR/Cas9 construct containing the gRNAs. After every GB reaction, during the screen-colonies step, different PCR were established to confirm the presence of the GBparts.

The screening of the multipartite GB reactions (assembly of pUPD_U626, gRNA and pUPD_scaffold) was conducted with a primer forward annealing before the LB (screening_α/Ω_for) and a primer reverse annealing after the gRNA (scaffold_rev). The resulting amplicon is 499 bp long (Figure 22a, b).

The first binary assembly was screened with a primer forward annealing on the AtU626 promoter (AtU6_for) and a revers annealing after the RB (Screen_rev). Two amplified regions are expected because AtU6_for anneals on the promoter of both gRNA1 and gRNA2, resulting in 742 bp and 388 bp amplicons (Figure 22c).

The second binary assembly, that leads the productions of the final plasmid, was screened with primer forward annealing before the LB (Screen_for) and the reverse on the scaffold (scaffold_rev), amplifying two regions: 830 bp and 1184 bp (Figure 22d).

The pDGB_α2_CcPDS (Figure 23) was examined with the Sanger sequencing. The result was aligned in Benchling (https://benchling.com/editor) to confirm the presence of all the GBparts.



	SEQUENCE
PF_gRNA1	ATTGGAGAAGAGAGGATCTGGAT
PR_gRNA1	AAACATCCAGATCCTCTCTTCTC
PF_gRNA2	ATTGGTCACCGATTGCAATTCCC
PR_gRNA2	AAACGGGAATTGCAATCGGTGAC

Figure 21. gRNAs sequences. The picture is a graphical representation of the CDS of *PDS*, with highlight of gRNA1 and gRNA2. The table includes gRNAs domesticated with "Domestication Tool" of GoldenBraid 2.0.

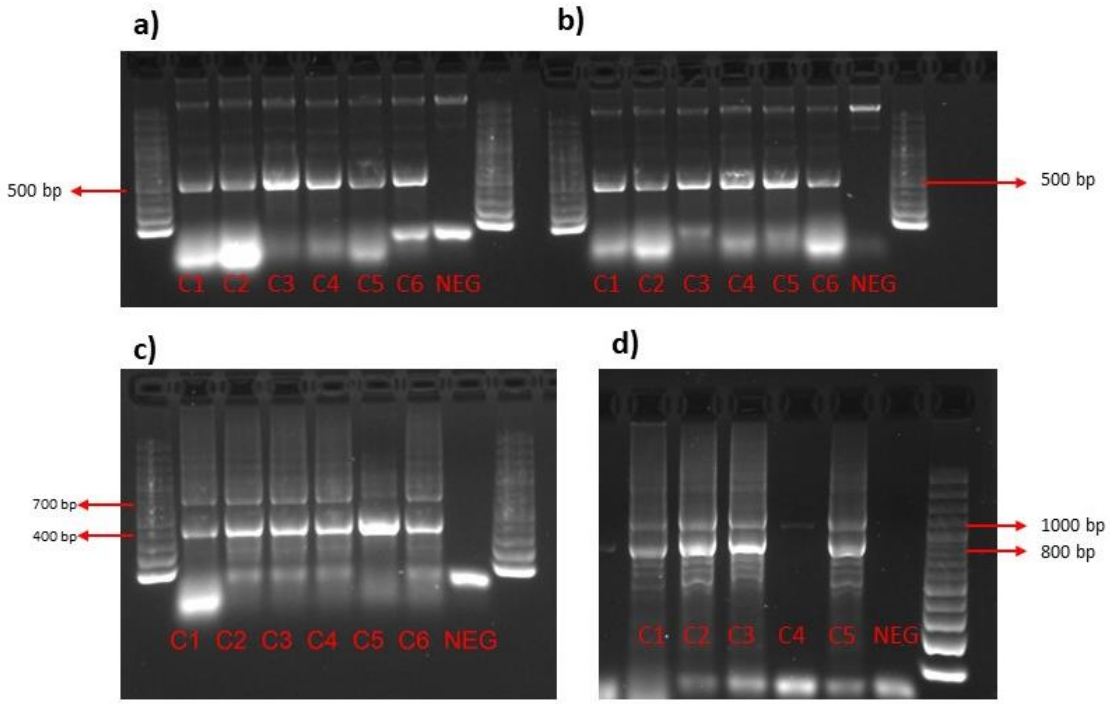


Figure 22. gels elettrophoresis of screen-colonies-PCR products. a) first screening on six *E. coli* colonies containing promoter U626, gRNA1 and scaffold. **b)** screen on six colonies containing promoter U626, gRNA2 and scaffold. **c)** screening of the second GB reaction containing gRNA1 and gRNA2 in a pDGB_Ω2. **d)** screening on the final pDGB_α2_Cc_PDS containing the gRNAs, the Cas9 and nptII sequences.

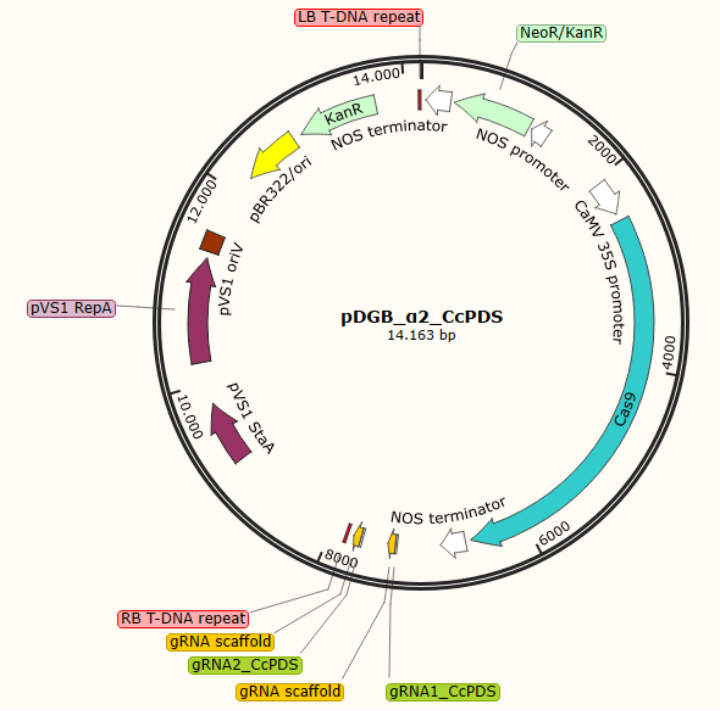


Figure 23. graphical representation of pDGB_α2_CcPDS.

3.6 Plant transformation

Transformation with vectors containing *GFP* reporter gene

Spinoso di Sardegna shoot apical meristems were transformed with microinjection and co-culture with GV3101 strains of *A. tumefaciens* containing two different constructs: pK7WGF2 and pGUS_INT.

After two days of *in vitro* culture in presence of *A. tumefaciens* the meristems were transferred in medium containing TIM (150 mg ml^{-1}) to eliminate *A. tumefaciens*. Three weeks after the transformation the meristems developed plantlets (Figure 24a)

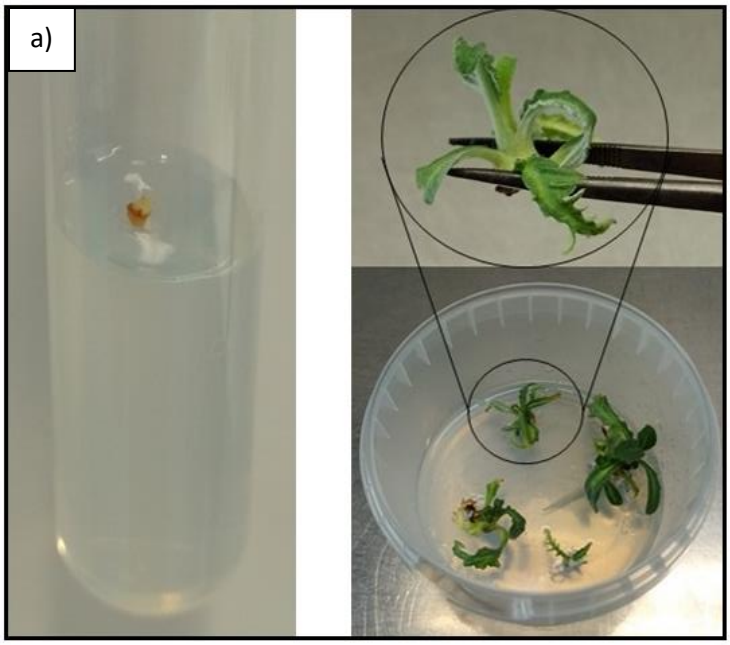
One week after the treatments the number of grown plantlets was established. For both plasmids used, the co-colture showed 10% more of grown meristems than micro-injection (see first table in Figure 24)

Transformation with pDGB_α2_CcPDS and pTRANS_221_6

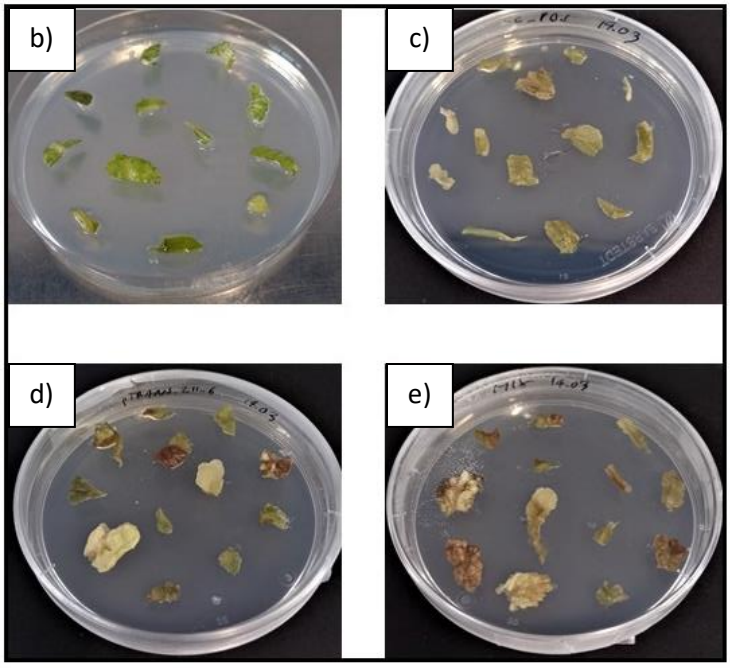
Romanesco C3 leaf explants (Figure 24b) were co-cultivated with *A. tumefaciens* with two different constructs: pDGB_α2_CcPDS contains a CRISPR/Cas9 system and two gRNAs targeting the globe artichoke *PDS*; pTRANS_221_6 contains *WUS* and *BBM* sequences, growth regulators expressed in SAM that can help the regeneration from the callus.

The two constructs were used both separately and mixed, to detect the effect of growth regulator in transformed explants (Figure 24c, d, e).

One month after the treatment different stadium of explants were evaluated: green leaves, dead leaves (brown), brown calli and green-white calli. Treatment with pTRANS_221_6 (both alone and mixed with pDGB_α2_CcPDS) showed 20-30% more callogenesis than treatment with pDGB_α2_CcPDS alone.



Plasmid	Treatment	TOT	% grown after 3 week
pGUS_INT	MI	9	67
	CC	22	77
pK7WGF2	MI	34	79
	CC	39	90
Control		4	100



Plasmid	TOT	Dead (%)	Green (%)	Calli	
				B (%)	GW (%)
pDGB_Cc_PDS	218	31	53	7	9
pTRANS_221_6	194	24	39	6	30
MIX	182	23	29	22	26
Controls	20	0	0	0	100

Figure 24. Plant transformation. a) Spinoso di Sardegna transformation. On the left it a shoot apical meristem before the infection is pictured. On the right the grown meristems after three weeks of in vitro culture. **b)** Romanesco C3 leaves explants after co-culture with *A. tumefaciens.* **c)** explants one month after transformation with: pDGB_Cc_PDS **d)** pTRANS_211_6 **e)** mix of two plasmids. The first table indicates per percentage of grown plantlets three weeks after the treatment. The second table represents different evolutions of leaf explant one month after treatment; TOT i.e. total number of explants, B i.e. brown calli and GW i.e. green-white calli.

4 DISCUSSION

The economic importance of globe artichoke, both for agri-food and pharmaceutical industry (especially as dietary supplement), opened interesting research perspectives in agronomic and biotechnological fields. Globe artichoke's antioxidant capacity is one of the highest in vegetables and it derives from its large content of polyphenols (chlorogenic acid, cynarin, luteolin 7-O-rutinoside, and luteolin 7-O-glucoside). However, due to polyphenol content and high expression of polyphenol oxidase (PPO), it shows tissue browning after wounding. This biochemical activity reduces the antioxidant capacity of the food product, resulting in a decrease of the nutritional value and the shelf life of the product. For these reasons the reduction of PPO's activity gained interest in scientific community. The common strategies to reduce this process, like blanching (heat treatment), immersion in ascorbic acid solutions or the use of packaging to reduce the contact with oxygen (that is necessary for the enzymatic reaction), can't solve the problem at all. Blanching has negative effects on flavor, texture and color; for this reason it can be replaced by the use of appropriate packaging (Moon et al., 2020). The chemical strategies aren't accepted by many consumers and can't be applied to intact vegetables and fruits (Whitaker J. R. et al., 1995). The food industry wants to meet the interests of the consumers by being more sustainable avoiding food waste (mostly caused by reduction of shelf life of products); however the strategy to control the tissue browning represent a cost in terms of energy, materials and used chemicals. Indeed the most efficient techniques for controlling the PPO's activity are based on the genetic engineering of one or more genes of this family. In last few years, research works studied different species of agronomic interest and set up protocols for genetic improvement aiming the inactivation of PPO's, resulting in reduction of enzymatic browning. The common mushroom (*Agaricus bisporus*) was modified using CRISPR/Cas9 system for a knock down of *PPO* (Waltz E., 2016) and the Artic® apple was obtained with RNA interference strategy designed on *MdPPO2* (Stowe & Dhingra, 2020). These methods could even be applied in globe artichoke to improve the quality.

In 2013, Quarta and colleagues studied for the first time a gene coding for a globe artichoke *PPO*. The expression level of this *PPO* resulted to be upregulated 48 hours after wounding, although the tissue browning was observed few minutes later. This result suggested the presence of other *PPO* genes involved in the globe artichoke browning process. As support to this hypothesis, thanks to the availability of the whole globe artichoke genome sequence (Acquadro et al., 2020; Scaglione et al., 2016), in this thesis a total of 11 *PPO* genes were isolated. Thus, in a view of improving qualitative properties in globe artichoke, in this thesis the *PPO* gene family has been molecularly characterized and putative functions have been associated through phylogenetic analysis and TFBSs identification. Moreover different transformation and regeneration protocols have been proposed.

In the first part of this thesis, bioinformatic and *in silico* analyses have been conducted to acquire information regarding *PPOs* genomic and transcriptional features. Structural genetic and protein profiles of the eleven *PPOs* were generated. The phylogenetic tree and the analysis of putative TFBSs called attention to some *PPOs* whose expression is regulated by light stimuli, wounding, and hormones (ethylene, jasmonic acid and salicylic acid) signaling and biosynthetic pathways involved in senescence and defense against biotic attacks (both these processes cause tissue browning) (Ahlawat & Liu, 2021; Ahmad et al., 2013). We confirmed the results obtained by Quarta and colleagues that, in 2013, analyzed the promoter sequence ("1432 bp 50 upstream of the translation start codon") of the *PPO7* and individuated putative cis-acting elements involved in response to light stimuli and wounding. Moreover, before starting with molecular analysis, the allelic variants of the eleven *PPOs*, were detected in four globe artichoke genotypes using v.2.0 genome as reference; a range of 600-700 SNP/Indel per variety was evaluated. The majority is represented by SNPs, while the rest is insertions and deletions. In Spinoso di Sardegna, Violetto di Sicilia and Violetto di Toscana five high impact SNP/Indel were pointed out, while in Romanesco C3 six were individuated. In SP, VS and C3 the same deletion of six bp in homozygosis was detected in the initial part of the sequence in *PPO8*. In VT the insertion of four bp in the initial part of *PPO5* is in homozygosis. These mutations probably affect the translation of the mRNA, forming unfunctional protein.

The second part of this thesis aimed at identifying those *PPOs* putatively associated to globe artichoke tissue browning. The expression levels of the eleven *PPOs* were evaluated by RTqPCR in different tissues (pre and post wounding) of VT variety and in different callus morphologies of SP variety. VT was chosen because of its high tissue browning after wounding while SP for the availability of efficient *in vitro* maintenance protocols. Transcriptional profiles revealed major levels of *PPOs* expression in secondary capitulum both at basal or upon wounding treatment. In calli tissues, an increasement in the expression of some *PPOs* was observed in brown calli compared to white and green calli. Differently from Quarta et al. (2013) that highlighted an up-regulation of *PPO7* 48h after wounding, our results highlighted the leaf-specificity of *PPO7*. We can thus hypothesize that the tissue browning immediately after cutting might be caused by other *PPOs* genes. Indeed, from statistical analysis it was possible to infer that *PPO6* and *PPO11* are the most implied in tissue browning, while *PPO1*, *PPO2* and *PPO5* are also probably involved but with a minor effect. Further works may focus on knock-out of *PPO6* and *PPO11* in globe artichoke with the aim of reducing tissue browning.

The third part of this thesis has been focused on advanced biotechnological approaches, like setting up of genetic transformation and gene-editing approaches, potentially useful in a view of globe artichoke genetic improvement. The development of new genome editing technologies in plant breeding has fostered a growing interest for in vitro culture and regeneration protocols, which represent a major bottleneck in the application of these techniques in recalcitrant species such as globe artichoke. According to literature, in 1990s,

globe artichoke calli were transformed with reporter genes for the first time, but no plant regeneration was obtained (Gonzales & Kchouk, 1994). In later years, the same authors performed particle bombardment on axillary buds, bypassing the organogenesis process (Kchouk et al., 1997). Since then, various protocols have been tested using different globe artichoke tissues and induction mediums, but regeneration was never obtained. An important factor responsible for the recalcitrance to *in vitro* regeneration is the tissue browning caused by phenolic compounds that leads to tissue necrosis (Dan Y., 2008). Within this framework, more efforts are needed with the aim of identifying an efficient transformation and regeneration protocol useful for globe artichoke genetic improvement. Here, different transformation procedures have been initiated using combinations of vectors containing reporter, marker or growth regulator genes. On a side, to skip the regeneration step, SAMs were transformed by *A. tumefaciens* with micro-injection and co-culture treatments using two constructs containing *GFP* and *GUS* reporter genes. Micro-injection of SAMs of cucumber plants previously performed (Baskaran P. et al. 2016); by testing different conditions and tissue explants the greater transformation efficiency was detected through SAM's micro-injection. In this thesis, compared to co-culture, the micro-injection led to a loss of 20% of plantlets, probably due to the invasiveness of the injection procedure as reported by Vinoth S. et al. (2013) who demonstrated that the microinjection can lead to a severe damage that cause the break of the meristems. The transformation efficiency hasn't been detected yet. On the other side, a strategy to help organogenesis in recalcitrant species is the ectopic expression of growth regulators involved in SAM regulation (Maher et al., 2020). Thus, here leaves explants were transformed by *A. tumefaciens* with co-culture using (independently or in combination) two constructs: one carrying the CRISPR/Cas9 system to obtain a loss-of-function of *PDS* gene, and one for the ectopic expression of *WUS* and *BBM* growth regulators. The explants transformed with the second cited construct showed 20% more callogenesis rate. Actually, the positive effects of *WUS* and *BBM* in regeneration steps of transformation protocols have been demonstrated in previous study. In pepper the transformation with *BBM* leaded to direct regeneration of this otherwise recalcitrant species (Heidmann I et al., 2011). Meanwhile Kadri Aline et al. (2021) established a genetic transformation with ectopic expression of *WUS* in *Medicago truncatula* resulting in increased callogenesis.

In conclusion, in this thesis a molecular and functional characterization of PPO gene family has been conducted in globe artichoke species; the acquired information will potentially allow to arrange further research works that aim at deepening the knowledge of this gene family, which is key for the quality of the product. Moreover, different transformation and regeneration protocols have been proposed. These protocols, although still in the process of optimization, may represent a first step toward the establishment of a successful *PPOs*-based genetic engineering improvement of globe artichoke species. *PPO6* and *PPO11* could be interesting

candidates for gene knock out, in order to reduce tissue browning and to improve *in vitro* regeneration. Since these genes are highly upregulated in brown calli their KO could lead to reduction in phenolic oxidation that hampers *in vitro* regeneration.).

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