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Plant degreening: evolution and expression of tomato (*Solanum lycopersicum*) dephytylation enzymes

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ABSTRACT

Chlorophyll is the most abundant pigment on earth and even though it is known that its high photo-excitability necessitates a tight regulation of its degradation pathway, to date there are still several steps in chlorophyll breakdown that remain obscure. In order to better understand the 'degreening' processes that accompany leaf senescence and fruit ripening, we characterized the enzyme-encoding genes involved in dephytylation from tomato (*Solanum lycopersicum*). A single pheophytinase (*PPH*) gene and four chlorophyllase (*CLH*) genes were identified in the tomato genome. A phenetic analysis revealed two groups of CLHs in eudicot species and further evolutionary analysis indicated that these enzymes are under diverse selection pressures. A comprehensive expression profile analysis also suggested functional specificity for these dephytylating enzymes. The integrated analysis allows us to propose three general roles for chlorophyll dephytylation: *i*) PPH, which is under high selective constraint, is responsible for chlorophyll degradation during developmentally programed physiological processes; *ii*) Group I CLHs, which are under relaxed selection constraint, respond to environmental and hormonal stimuli and play a role in plant adaptation plasticity; and *iii*) Group II CLHs, which are also under high selective constraint, are mostly involved in chlorophyll recycling.

1. Introduction

Chlorophyll (Chl) is responsible for light absorption during photosynthesis, a process upon which life on Earth depends. Chl is composed of a tetrapyrrole ring with a central magnesium ion and a phytol chain and its metabolism can be divided into three phases: biosynthesis, the chlorophyll cycle, and degradation. However, in part due to its complex biochemical structure, various aspects of Chl metabolism are still poorly understood, and this is especially true for those associated with its degradation (Hörtensteiner, 2013). In general, the Chl degradation pathway resembles bacterial detoxification processes, and for many years it was considered to be a simple Chl detoxification phenomenon (Hörtensteiner, 2006). However, recent studies indicate that the process is more complicated since more Chl derived catabolites are being discovered, although few of these have been assigned a biological function (Hörtensteiner, 2013; Hörtensteiner and Kräutler, 2011).

Chl breakdown sequentially involves a dephytylation step, tetrapyrrole ring opening and catabolite inactivation to avoid phototoxicity of Chl degradation products, resulting in a linear tetrapyrrole molecule that is stored in the vacuole. For many years, it was accepted that dephytylation was catalyzed by chlorophyllase (CLH), an enzyme that hydrolyzes Chl *a* to generate phytol and chlorophyllide (Chlide *a*), a compound that is further converted to pheophorbide *a* (Pheide *a*) by a still unknown metal dechelatase. The enzyme pheophorbide *a* oxygenase (PAO) then opens the tetrapyrrolic backbone structure, producing the red chlorophyll catabolite (RCC) that follows the "PAO pathway" of Chl breakdown until catabolite inactivation (Hörtensteiner, 2013). However, data obtained from experiments with *Arabidopsis thaliana* double mutants demonstrated that CLHs are not required for senescence-related Chl breakdown in vivo (Schenk et al., 2007). Furthermore, subcellular localization analyses have resulted in contradicting observations since *Arabidopsis* CLHs have been reported to be cytoplasmic proteins (Schenk et al., 2007), while it was demonstrated that a *Citrus* CLH resides in the plastid and is post-transcriptionally

Abbreviations: ABA, abscisic acid; Chl, chlorophyll; Chlide, chlorophyllide; CLH, chlorophyllase; *dN*, non-synonymous distance; *dS*, synonymous distance; *gf*, green flesh mutant; LTR, likelihood ratio test; MS, Murashige and Skoog media; Nc, number of codons; PAO, pheophorbide a oxygenase; PAR, photosynthetically active radiation; pFCC, primary fluorescent chlorophyll catabolite; Pheide, pheophorbide; Pheo, pheophytin; PPH, pheophytinase; RCC, red chlorophyll catabolite; SAG12, senescence associated gene 12; SE, standard error; SGR, stay green protein; UTR, untranslated regions.

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regulated by cleavage (Azoulay-Shemer et al., 2008, 2011; Harpaz-Saad et al., 2007). This discrepancy might reflect a difference in experimental approaches, but this issue remains to be resolved. In 2009, Schelbert et al. identified an *A. thaliana* plastidial protein that dephytylates the Mg-free Chl *a*, pheophytin (Pheo *a*), yielding phytol and Pheide *a*. This new dephytylating enzyme was named pheophytinase (PPH) and shown to be associated with the thylakoid membrane as part of a Chl breakdown complex recruited by the STAY-GREEN protein (SGR). Interestingly, this degradation complex involves all the enzymes that catalyze the stepwise degradation of Chl to the compound known as primary fluorescent chlorophyll catabolite (pFCC), allowing metabolic channeling of phototoxic Chl breakdown intermediates. Subsequently, pFCC is exported from the plastid to the vacuole (Sakuraba et al., 2013). Interestingly, CLH is not part of this enzymatic complex (Sakuraba et al., 2012), suggesting different and/or complementary roles for the two dephytylation enzymes.

Chl degradation has been extensively investigated and most studies to date have used leaf senescence as the associated experimental system. Leaf senescence is a highly regulated process that involves several changes in cell structure, metabolism, and gene expression. The earliest and most evident cell structure change is the breakdown of the chloroplast, accompanied by catabolism of chlorophyll and macromolecules (e.g. proteins, lipids, and RNA). Regarding gene expression, leaf senescence is characterized by the decrease of genes related to photosynthesis and protein synthesis and by increased expression of senescence-associated genes (SAGs) (Lim et al., 2007). Senescence is an integrated response of plants to endogenous developmental and external environmental stimuli, and plant hormones play an important role in signaling. Our current understanding of the relationship between environmental responses and leaf senescence comes from the study of senescence response to the phytohormone abscisic acid (ABA), involved in response to abiotic stresses such as drought, high salt condition, and low temperature and; jasmonic acid (JA) and salicylic acid (SA) that participate in biotic stress regulation. Meanwhile, ethylene has extensively been described as a major hormone in hastening age-dependent leaf senescence (Khan et al., 2013). However, relatively little is known about other examples when degreening occurs, such as the mechanisms of Chl degradation that accompany fruit ripening (Hörtensteiner, 2013). Fleshy fruit ripening involves major biochemical and physiological alterations that influence texture, flavor and aroma as well as color (Seymour et al., 2013). This complex metabolic process is mainly controlled by ABA and ethylene. In the climacteric fruits such as tomato, there is an increase in ABA preceding the increase in ethylene. Exogenous application of ABA induces ethylene through the transcriptional activation of the biosynthesis genes, while the suppression of ABA leads to a delay in fruit ripening (McAtee et al., 2013). Additionally, this hormone has also been associated with fruit growth in tomato. ABA accumulates at the end of the expansion phase, and ABA-deficient mutants have reduced fruit size (Nitsch et al., 2012). In this framework, the cultivated tomato, *Solanum lycopersicum*, whose fruit exhibits clear color changes during maturation, is a potentially valuable model for comparative studies of degreening during leaf senescence and fruit ripening in relationship with their hormonal control. To our knowledge, the only report describing Chl degradation-associated genes in *S. lycopersicum* (Efrati et al., 2005) involved the mapping of three *CLH* genes and a *PAO* gene as part of a search for candidates for the *gf* (*green flesh*) mutant phenotype, which was later demonstrated to be SGR (Barry et al., 2008).

In this current study, we performed a genomic and evolutionary characterization of tomato *CLH* and *PPH* genes, in order to gain a more comprehensive understanding of their role in Chl dephytylation. Additionally, the expression patterns of these genes in leaves submitted to different hormonal treatments and during fruit development and ripening were analyzed. Our data demonstrate that the diversity of dephytylating enzymes is associated with different selective constraints and expression profiles, suggesting functional specialization.

2. Materials and methods

2.1. Phenetic and evolutionary analyses

Tomato *CLH* and *PPH* gene sequences were identified by using the coding sequence of both *A. thaliana CLH* genes (AT1G19670 and AT5G43860) and the *PPH* gene (AT5G13800) to interrogate the tomato genome sequence, available at the Sol Genomics Network (http://solgenomics.net/). The chromosomal locations were determined by the position of the closest marker, based on the Tomato-EXPEN-2000 map (http://solgenomics.net/). In silico prediction of the protein subcellular localization was performed using TargetP (Emanuelsson et al., 2007), Cello v2.5 (Yu et al., 2006), SherLoc2 (Briesemeister et al., 2009), MultiLoc2 (Blum et al., 2009), BaCelLo (Pierleoni et al., 2006), Plant-mPLoc (Chou and Shen, 2010), ChloroP (Emanuelsson et al., 2007) and iPSORT (Bannai et al., 2002). Other plant sequences that are homologous to the *A. thaliana CLHs* and *PPH* genes were identified by searching the Phytozome 9.0 database (http://www.phytozome.net/), NCBI (http://www.ncbi.nlm.nih.gov) and Sol Genomics Network, using the tBLASTx program (Altschul et al., 1990). The sequences were aligned using the MUSCLE package available in the MEGA 5.2 software with default parameters (Tamura et al., 2007) in a codon-based manner and inspected manually. The alignment was analyzed using the Neighbor-Joining method, the distances were calculated according to the best model pointed by MEGA 5.2 software and the tree topology was evaluated with 5000 bootstrap replications. Detailed information of all sequences used for the analyses is shown in Table S1.

The evolutionary analysis was performed as previously described by Almeida et al. (2011). Non-synonymous (*dN*) and synonymous (*dS*) distances, as well as their standard error (SE) values were estimated by the Nei–Gojobori method (p-distance) using the MEGA 5.2 software. In order to preserve the reading frames, the alignment gaps were deleted. Alignments are presented in Figs. S1 to S10. Codon bias was determined by the effective number of codons (Nc) value computed by the CodonW program (mobyle.pasteur.fr/cgi-bin/portal.py?-form¼codonw). The Nc value varies from 21, when only one codon is used per amino acid, to 61, when synonymous codons for each amino acid are used at similar frequencies. One-way ANOVA with Tukey's posthoc test was performed using the InfoStat software (www.infostat.com, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) to evaluate significant differences in codon usage. In order to compare codon evolution models to determine selective constraint, three models were fitted using the CODEML program of the PAML suite (Yang, 2007). The first model, M0, assumes that all codons across the sequences have the same dN/dS ratio (ω). The value for ω provides an indication of the selection at the protein level: $0 < \omega < 1$ indicates purifying selection; $\omega = 1$ is neutral evolution; and $\omega > 1$ points to the presence of positive selection. Model M1a suggests the existence of two classes of codons, a proportion with $0 < \omega < 1$ and the remainder of codons with $\omega = 1$. Model M2a indicates three types of codon evolution: purifying selection, neutral evolution, and positive selection. The fit of models M0 versus M1a, and M1a versus M2a was evaluated by a likelihood ratio test (LRT), comparing the difference in log likelihoods with a χ^2 distribution two times (Yang, 2007).

2.2. Plant material

S. lycopersicum L. (cv. MicroTom) seeds were obtained from the Laboratory of Hormonal Control of Plant Development (ESALQ, Universidade de São Paulo). The plants were grown in 1 L pots in a greenhouse under automatic irrigation (four times a day) at an average mean temperature of 25 °C, 11.5 h/13 h (winter/summer) photoperiod and 250–350 μmol m^{-2} s^{-1} of incident photo-irradiance. Fruit pericarp material (without placenta and locule walls) at the green (G, ~1.5 cm in diameter), mature green (MG, jelly placenta and ~2.5 cm in diameter),

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