Identification and Cloning of Differentially Expressed SOUL and ELIP Genes in Saffron Stigmas Using a Subtractive Hybridization Approach

Oussama Ahrazem\(^1\)\(^2\), Javier Argandoña\(^1\), Raquel Castillo\(^3\), Ángela Rubio-Moraga\(^1\), Lourdes Gómez-Gómez\(^1\)\(^*\)

\(^1\) Instituto Botánico, Departamento de Ciencia y Tecnología Agroforestal y Genética, Facultad de Farmacia, Universidad de Castilla-La Mancha, Albacete, Spain, \(^2\) Facultad de Ciencias Ambientales y Bioquímica, Universidad de Castilla-La Mancha, Toledo, Spain, \(^3\) VITAB Laboratorios, La Gineta, Albacete, Spain

\(^*\) Marialourdes.gomez@uclm.es

### Abstract

Using a subtractive hybridization approach, differentially expressed genes involved in the light response in saffron stigmas were identified. Twenty-two differentially expressed transcript-derived fragments were cloned and sequenced. Two of them were highly induced by light and had sequence similarity to early inducible proteins (ELIP) and SOUL heme-binding proteins. Using these sequences, we searched for other family members expressed in saffron stigma. ELIP and SOUL are represented by small gene families in saffron, with four and five members, respectively. The expression of these genes was analyzed during the development of the stigma and in light and dark conditions. ELIP transcripts were detected in all the developmental stages showing much higher expression levels in the developed stigmas of saffron and all were up-regulated by light but at different levels. By contrast, only one SOUL gene was up-regulated by light and was highly expressed in the stigma at anthesis. Both the ELIP and SOUL genes induced by light in saffron stigmas might be associated with the structural changes affecting the chromoplast of the stigma, as a result of light exposure, which promotes the development and increases the number of plastoglobules, specialized in the recruitment of specific proteins, which enables them to act in metabolite synthesis and disposal under changing environmental conditions and developmental stages.

### Introduction

The saffron stigma accumulates huge quantities of soluble apocarotenoids, crocins and picrocrocin up to the time the floral bud emerges from the soil. These components then remain relatively constant until flower senescence, when they begin to decrease [1]. These changes are transcriptionally coordinated with a parallel regulation of genes from the carotenoid pathway and with the carotenoid cleavage dioxygenase CsCCD2 gene, involved in the formation of these apocarotenoids [2,3,4]. The synthesis of crocins takes place inside the chromoplasts from...
the oxidative cleavage of zeaxanthin (Fig 1) [5], and the differentiation from amyloplasts to chromoplasts is concomitant with massive crocin biosynthesis during stigma development [5]. Once synthesized, crocins pass from the chromoplast to the vacuoles where they accumulate, and chromoplast ultrastructure starts changing with an increasing accumulation of plastoglobules and empty vesicles [6]. Interestingly, the increased number of plastoglobules is accompanied by transcriptional up-regulation of another carotenoid cleavage enzyme, CsCCD4a/b, localized in the plastoglobules, and involved in the formation of a different apocarotenoid in the stigma, β-ionone, produced at high levels in preanthesis and anthesis stigmas [1,7].

Light is one of the most crucial environmental factors influencing carotenoid accumulation in plants, principally due to their role in protecting the photosynthetic apparatus against excess light [8]. The light-mediated regulation of the carotenoid pathway implies transcriptional control of genes encoding phytoene synthase (PSY) by components of the light signaling pathway as the transcription factors of the bHLH phytochrome-interacting factor (PIF) family [9,10], and the bZIP transcription factor HY5 [11]. In addition, light also increases carotenoid accumulation in chromoplast containing tissues as in some citrus species and tomato [12]. In fact, white tomato fruits that lack carotenoids are obtained by avoiding light exposure from the early stages of fruit development [13]. However, an opposite effect is observed when grapefruits are covered at the immature green stage. These exhibit an accelerated accumulation of carotenoids compared to light-exposed fruits [14], an effect also observed in certain tomato varieties [15]. An accumulation of carotenoids is also a characteristic of certain organs and subterranean tissues that accumulate carotenoids in the absence of direct light, as in the case of orange carrots or in yellow sweet potato, cassava and taro [16,17,18]. When carrot roots are illuminated, their carotenoid profile is similar to those obtained from leaves, while chromoplast development is prevented and chloroplasts are developed [17].

During the development of saffron stigmas, CsCCD2 expression and crocin accumulation are down-regulated by light [4]. The earliest developmental stages of the flower in saffron take place beneath the soil surface (Fig 1), and during these stages crocins are accumulated at high levels in the stigma, due to the transcriptional activation of the carotenogenic genes and increased expression of CsCCD2 [1,4]. As the flower reaches its mature stage, the expression of the carotenogenic genes is downregulated with no more net accumulation of crocins, and shortly after the fully developed flower emerges from the soil to the light (Fig 1). At this time, other apocarotenoid compounds are generated by the stigma. The production of β-ionone and other apocarotenoid volatiles seems to be induced by light in stigmas at preanthesis and anthesis [1], suggesting a complex apocarotenoid modulation by light in the development of the stigma and in carotenoid metabolism. Because of the limited molecular information concerning the regulation of apocarotenoids under different light conditions, the objective here is to improve our knowledge about the mechanisms of the transduction pathway of light-controlled apocarotenoid biosynthesis which could be helpful for programs aiming to increase the apocarotenoid content in saffron stigmas.

Suppression subtractive hybridization (SSH) is a powerful technique for the identification of differentially expressed genes, including those genes present in relatively low abundance [19]. We used SSH to isolate and characterize expressed sequence tags (ESTs) produced in red stigmas in response to light exposure during 24 hours versus stigmas submitted to 24 hours of darkness. Among the identified cDNA fragments, two of them showed the highest levels of induction by light and were chosen for further analyses and characterization. The work presented here shows the differential accumulation of several ELIP and SOUL proteins in saffron stigmas during the transition from dark to light conditions. During this transition, stigmas develop an intricate membrane network and a high accumulation of plastoglobules is observed, suggesting a function for ELIP and SOUL proteins in the reorganization of saffron chromoplasts.
**Results**

**Identification of specific transcripts induced by light**

Differential subtractive hybridization analysis was carried out and 22 clones were finally sequenced. Expression of these genes in light and dark treated stigmas was verified using qRT-PCR. A total of 22 clones were clearly up-regulated by light (Table 1). Several clones were associated to light responses. Clones LD3029 and LD3545 showed identity to the Arabidopsis nuclear-encoded sigma factor 5 (SIG5), which controls circadian rhythms of transcription of several chloroplast genes [20]; LD3750 showed identity to bZIP transcription factor Elongated Hypocotyl 5 (HY5), a critical positive regulator of light responses [21]; LD 3535 showed identity to HYH, predominantly involved in blue-light regulation, and whose function in part overlaps with that of HY5 [22]. LD3644 showed identity to ARF protein (Attenuate Far-Red response), which plays a positive role in phytochrome A (phyA)-mediated light signaling in Arabidopsis. LD3685 showed identity to early light-induced proteins (ELIPs), nuclear-encoded thylakoid membrane proteins that are transiently expressed immediately after light stress [23], and finally LD3572 with identity to SOUL/HBP proteins with a role in light signaling in vertebrates [24]. Among them, the expression of LD3572 and LD3685 was significantly induced by light (Table 1), and both clones were further analyzed.

**Identification of saffron ELIPs**

An ELIP cDNA fragment was identified as being highly induced by light (Table 1). Using this sequence several putative *Crocus sativus ELIP* sequences were identified in the yellow and
orange stigma transcriptomes of saffron [4]. Four different full-length cDNA clones were finally identified (Genbank ELIPa, KX374537; ELIPb, KX374538; ELIPc, KX374539; ELIPd, KX374540). Both, ELIPa and ELIPb ORFs encode deduced polypeptides of 184 amino acids, with a predicted molecular mass of 19.37 kDa. ELIPc ORF encodes a deduced polypeptide of 188 amino acids, with a predicted molecular mass of 19.86 kDa, and ELIPd ORF encodes a deduced polypeptide of 172 amino acids, with a predicted molecular mass of 17.97 kDa. Main differences among the ELIP proteins were present in the N-t domain (Fig 2A).

ELIPa and ELIPb shared 97.27% identity, while ELIPc and ELIPd shared 78.49% identity. All the ELIP proteins from saffron were predicted to be targeted to plastids by using the TargetP 1.1 programme (http://www.cbs.dtu.dk/services/TargetP) and to contain three similar hydrophobic domains (Fig 2B) that allowed their insertion into the membrane [25]. The three transmembrane helices of 19–23 amino acids each (Fig 2A and 2B) are also present in ELIPs from pea, barley and Arabidopsis [26]. Helices I and III of ELIPs in saffron share the Glu (E), Arg (R), and Asn (N) residues involved in binding the four core chlorophylls in Lhcb1 [27] [28]. The second Glu (E) residue located in the C-terminal region of helix III (Fig 2A) is conserved in all known eukaryotic and prokaryotic ELIPs. Furthermore, helices I and III of ELIPs contained a conserved LAM(GAM)FAM motif (Fig 2A).

The data reported for ELIPs from pea, barley and Arabidopsis predicted that these proteins are type II membrane proteins with the N-terminus on the stromal and the C-terminus on the lumenal side of the thylacoidal membrane. The absence of chloroplasts in saffron stigmas,

### Table 1. EST clones up-regulated by light in red saffron stigmas.

| EST code | Sequence ID in the stigma transcriptome | L/D fold increase | Description | E-Value |
|----------|----------------------------------------|------------------|-------------|---------|
| LD3466   | gnl|SRA|SRR1140761.961746.2 | 4.515 | AT2G33690, Late embryogenesis abundant protein, group 6 | 2.00E-07 |
| LD3223   | gnl|SRA|SRR1767302.12929090.2 | 4.049 | No hit | |
| LD3220   | gnl|SRA|SRR1767302.21435612.1 | 4.022 | No hit | |
| LD3535   | gnl|SRA|SRR1767302.21770501.1 | 5.345 | HYH, AT3G17609 | 5.00E-22 |
| LD3572   | gnl|SRA|SRR1767302.22850438.2 | 7.238 | SOUL1, AT2G37970.1 | 3.00E-73 |
| LD3684   | gnl|SRA|SRR1767302.2144076.1 | 4.056 | No hit | |
| LD2408   | gnl|SRA|SRR1767302.21346505.2 | 4.251 | AT3G07230, MYB-like protein | 0.003 |
| LD3773   | gnl|SRA|SRR1767302.21176972.2 | 4.521 | dihydrolipoamide-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 1, AT5G55070 | 2.00E-33 |
| LD2637   | gnl|SRA|SRR1767302.11881954.2 | 4.015 | No hit | |
| LD3787   | gnl|SRA|SRR1767302.20570624.1 | 4.459 | No hit | |
| LD3742   | gnl|SRA|SRR1767302.22779218.2 | 5.207 | No hit | |
| LD2491   | gnl|SRA|SRR1767302.19318770.1 | 5.001 | BTB/POZ domain-containing protein NPYS, AT4G37590 | 2.00E-32 |
| LD3029   | gnl|SRA|SRR1767302.22801613.2 | 4.183 | SIGE AT5G24120, RNA polymerase sigma factor sigE | 3.00E-25 |
| LD2434   | gnl|SRA|SRR1767302.14379052.1 | 4.485 | No hit | |
| LD3685   | gnl|SRA|SRR1767302.13910891.1 | 13.910 | ELIP1 P93735 | 2.00E-34 |
| LD2905   | gnl|SRA|SRR1767302.5420811.1 | 4.036 | 40S ribosomal protein Q0IXR7 | 1.00E-15 |
| LD3658   | gnl|SRA|SRR1767302.22420952.2 | 4.285 | 3’-N-debenzyloxy-2’-deoxytaxol N-benzoyltransferase-like XP_010911309.1 | 7.00E-125 |
| LD3018   | gnl|SRA|SRR1767302.17072756.2 | 4.684 | No hit | |
| LD3002   | gnl|SRA|SRR1767302.21903636.2 | 4.048 | No hit | |
| LD3545   | gnl|SRA|SRR1767302.22144076.1 | 4.966 | SIGE AT5G24120, RNA polymerase sigma factor sigE | 2.00E-06 |
| LD3750   | gnl|SRA|SRR1767302.22918903.2 | 4.678 | transcription factor HY5-like XP_009381123.1 | 9.00E-51 |
| LD3644   | gnl|SRA|SRR1767302.21610532.2 | 3.259 | AFR Attenuated Far-Red Response XP_009387460.1 | 1.00E-111 |

doi:10.1371/journal.pone.0168736.t001
Fig 2. Comparison and analyses of ELIP proteins. (A) Amino acid alignment of the four ELIP proteins isolated from saffron stigmas. Conserved residues are depicted with asterisk. The colors of the residues reflect their physicochemical properties: red for small (small+ hydrophobic (incl. aromatic -Y)); blue, acid; magenta, basic—H; green, Hydroxyl + sulfhydryl + amine + G. (B) Tridimensional models of ELIPA-d and their disposition in the membrane. (C) Phylogenetic analysis of ELIP proteins. An un-rooted phylogenetic tree was
which only contain chromoplasts with a network of tubular membranes and vesicles inside, did not allow us to extrapolate this prediction to the ELIPs from saffron.

**Phylogenetic analysis of saffron ELIPa-d**

To examine the relationship of ELIPa-d with ELIP-like proteins from other plant species, the deduced amino acid sequences were analyzed, and an un-rooted Neighbor-Joining tree was built up (Fig 2C). The relationships shown in this gene tree reflect standard groupings of monocots and dicots, with clear separation of grass-sequences from the other monocot proteins.

**Saffron ELIPs mRNA are differentially regulated during stigma development**

The presence of four ELIP proteins in saffron raised the question about their redundant physiological function. We investigated their expression levels throughout the development of the stigma (Fig 3A). All of the four ELIP genes showed relatively low levels of expression in the white, yellow, orange and red stigmas (Fig 3A). However, ELIPa, ELIPb and ELIPc showed different expression patterns during these early developmental stages. All these stages took place under the soil, in dark conditions. ELIPa showed an increased expression in the yellow stage and its expression decreased thereafter (Fig 3A). ELIPb expression was already detected in the white stage and increased in the yellow and orange stages (Fig 3A). ELIPc expression levels increased from the white up to the orange stage and decreased thereafter in the red stage (Fig 3A). Under the conditions tested ELIPd transcripts were practically undetectable in all the selected developmental stages. In the following developmental stages, from preanthesis to post-anthesis the flowers had already emerged from the soil and were exposed to light. There was a marked peak of abundance of ELIPa-c mRNAs in the postanthesis stage (Fig 3A), with ELIPc being the gene with the higher expression levels.

**Expression levels in different tissues and light conditions**

In general, light plays a key role in ELIP expression levels in plants. Therefore, we analyzed how transcripts levels of ELIPa-d were influenced in red stigmas that were kept in continuous light or dark conditions during 24 hours. Continuous light induced the expression of all ELIP genes (Fig 3B), although at very different levels, with ELIPa showing the highest levels of induction, followed by ELIPc (Fig 3B). ELIPb and ELIPd showed a lower increase, suggesting different roles of ELIP genes in response to light. Further, since ELIP genes have been shown to be induced by different abiotic stimuli in several plant species [29,30], we sought to determine whether the ELIPa-d transcripts can be stimulated by abiotic stresses, the expression patterns of ELIPa-d in response to the plant hormone ABA, hyper-osmotic stress by NaCl, heat and dehydration were evaluated by qRT-PCR (S1 Fig). Compared to the control, ELIPa and ELIPc showed the highest induction levels due to the different treatments, with ELIPc transcript levels being significantly up-regulated in all the conditions tested.

Expression levels of ELIPa-d were also analyzed in leaves, corms, cormlets, roots, tepals and stamen. In leaves, ELIPd showed the highest expression levels suggesting a preferred leaf-
Fig 3. Expression analyses of ELIP genes isolated from saffron. (A) Relative expression levels of ELIPa-b during the development of the stigma in saffron. Inset are shown the relative expression levels of ELIPa-b in the early undeveloped stages of the stigma tissue. (B) Relative expression levels of ELIPa-b under two different light conditions and in several saffron tissues. Inset are shown the expression levels of these genes in root, cormlet, corm, tepal and stamen. Error bars depict SD. Below are shown the pictures of stigmas at different developmental stages: white, yellow, orange, red and stigmas at anthesis.

doi:10.1371/journal.pone.0168736.g003
specific expression of this gene in saffron (Fig 3B). The expression levels of ELIPA-d in the other tissues tested were low, with an undetectable expression of ELIPb in subterranean organs (corm, cormlet and roots) (Fig 3B).

SOUL proteins in saffron stigmas

The cDNA fragment with homology to SOUL proteins was used for the identification of SOUL homologues in saffron stigmas as performed for ELIP clones. Five SOUL-like genes were identified and named as SOULa-e (Genebank numbers: SOULa, KX374541; SOULb, KX374542; SOULc, KX374543; SOULd, KX374544; SOULe, KX374545) (Fig 4A). SOULa encodes a protein of 293 amino acids with a predicted molecular mass of 33.34 KDa. SOULb ORF encodes a deduced polypeptide of 206 amino acids, with a predicted molecular mass of 22.55 kDa. SOULc ORF encodes a polypeptide of 216 amino acids, with a predicted molecular mass of 24.45 kDa; SOULd ORF encodes a polypeptide of 240 amino acids, with a predicted molecular mass of 26.73 kDa, and finally SOULe ORF encodes a polypeptide of 388 amino acids, with a predicted molecular mass of 44.03 kDa. The SOUL proteins from saffron showed relatively low identities. SOULc and SOULd localization were assigned to secretory pathways using TargetP 1.1 and SMART. SOULa and SOULe were predicted to be localized in plastids. However, no prediction could be made for SOULb. The five SOUL proteins were modeled based on crystal structure of human SOUL protein c3rkb [31]. The structure of all SOUL proteins consists of a central core containing stranded antiparallel β-sheets arranged in a distorted barrel, flanked by two α-helices (Fig 4B). SOULa, b, d and e seem to be able to interact with membranes with a depth/hydrophobic thickness of 4.6 ±0.5 Å, 7.4±0.2 Å, 3.5±0.5 Å and 5.4±0.6 Å, respectively, while SOULc seems to be soluble (Fig 4B).

Phylogenetic analysis in plants grouped the SOUL proteins into five clades based on sequence homology. Sequences in clades 1 and 2 are predicted to be localized in chloroplasts. In addition, sequences in clade 1 contained an N-terminal domain belonging to the nuclear factor 2-like superfamily (NTF2-like) present in other proteins [32]. SOULe was identified inside group 1 and SOULa was identified inside group 2. SOULb was present in clade 3, and no prediction for sub-cellular location was made for sequences from this clade. SOULc was present in clade 4 and SOULD in clade 5. Proteins in clade 5 were assigned to the secretory pathway. SOUL proteins from Arabidopsis and Chlamydomonas were also included in the phylogenetic tree for comparison and group determination.

SOUL expression during stigma development

qRT-PCR was performed in order to determine differences in the expression pattern of the identified SOUL encoding genes during the development of the stigma. SOULa and b showed a similar expression pattern, with a maximum at anthesis. SOULa showed a sinusoidal expression pattern, an increased expression in the yellow stage followed by a down regulation and thereafter increasing again from the red stage to the anthesis stage (Fig 5A). SOULb followed the same pattern although its expression levels showed more dramatic changes from the pre-anthesis to the anthesis stage (Fig 5A). SOULc showed two peaks of expression, and reached its highest expression levels in the red stage. By contrast, SOULD transcript levels continuously increased from the yellow stage until reaching its highest expression at the postanthesis stage (Fig 5A). SOULE levels were only detected in the earliest developmental stages, coincident with undeveloped flowers that remain under the soil in the dark, and the transcript levels were undetectable from preanthesis to postanthesis (Fig 5A).
Fig 4. Comparison and analyses of SOUL proteins from saffron. (A) Amino acid alignment of the five SOUL proteins isolated from saffron stigmas. Conserved residues are depicted with asterisk. The colors of the residues reflect their physicochemical properties: red for small (small+ hydrophobic (incl. aromatic -Y)); blue, acid; magenta, basic—H; green, Hydroxyl + sulfhydryl + amine + G. (B) Tridimensional models of SOULa-e and their disposition in the membrane. (C) Phylogenetic analysis of SOUL proteins. An un-rooted phylogenetic tree was constructed using MEGA6 software with the neighbor-joining method based on ClustalW multiple alignments. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2,500 replicates) are shown next to the branches. The sequences isolated in this study are grouped in the red sub-tree.

doi:10.1371/journal.pone.0168736.g004
The expression of SOULa-e was also analyzed in other tissues, and in red stigmas kept during 24 hours in continuous light or dark conditions. Overall SOULa-d showed their maximum expression levels in stigmas from fully developed flowers, and SOULE showed the highest expression levels in leaves (Fig 5B). SOULb exhibited high expression levels in tepals, and was also induced under light conditions (Fig 4B). However, the other SOUL genes were not significantly affected by the light conditions (Fig 5B). The expression levels of these genes were also tested under different stress conditions (S1 Fig).
Expression analyses of HY5 during the development of the stigma

It is known that the ELIP transcripts are induced by the HY5 protein in Arabidopsis [33]. The identification of homologues of both proteins in our SSH approach led us to follow up the expression of HY5 throughout the development of the stigma in order to shed light on the relationship between this protein and the different ELIP proteins identified in saffron. The expression levels of HY5 were very low during the initial developmental stages, from the white up to the red stages (Fig 6). However, in the following developmental stages, from preanthesis to postanthesis there was a marked increase in HY5 expression levels (Fig 6).

Discussion

Saffron flowers must quickly respond to changes in environmental conditions during their development. Flower initiation takes place below ground in the darkness and once all the flower parts are almost fully developed is when the elongated stalk emerges to the soil surface where the bud is ready to open [5]. Two processes associated to chromoplasts are evident during the development of the stigma. First, the accumulation of crocins in the undeveloped stigma while flowers remain under the soil in dark conditions, reaching the highest levels in the red stage [1], and second, the cessation of crocins accumulation and a rapid increase in the number of plastoglobules from the red stage onwards, when flowers are exposed to light [6,34].

Investigation of differential gene expression in red saffron stigmas kept during 24 hours in light or dark conditions may lead to the discovery of candidate genes associated to these processes in this tissue. In this study, 22 differential expression genes were identified by suppression subtractive hybridization combined with qRT-PCR. Several clones were identified as good candidates involved in light signaling and responses, including homologues to ELIP and SOUL proteins. These homologues were used to identify other members of the family in saffron and analyzed their expression throughout the development of the stigma, in different conditions and in other tissues.
The ELIP family in saffron stigmas

ELIP are nuclear-encoded proteins present only in pro- and eukaryotic photosynthetic organisms that were first identified in pea as a chloroplast membrane-targeted product, transiently expressed, and present at the earliest stages of greening after an etiolated-to-light transition [12, 13]. At the amino acid level, members of the ELIP family are closely related to light-harvesting chlorophyll a/b-binding (Cab) antenna proteins of photosystem I and II. In higher plants they are divided into three groups, depending on the number of transmembrane helices present in their tertiary structure. With one helix are the Hlips (high light-induced proteins), Ohps (one-helix proteins), and Scps (small Cab-like proteins); Seps (stress-enhanced proteins) called also Lils (light-harvesting-like) with two helices and ELIP represented by three helices in the structure [35]. The light-induced ELIP protein identified in the suppression subtractive experiment in saffron stigmas belongs to the ELIP group. Although four genes encoding for ELIP proteins were isolated in saffron stigmas, we cannot exclude the presence of additional ELIP proteins expressed in other tissues. The number of ELIP genes is quite variable among plant species (https://phytozome.jgi.doe.gov). In the genome of Eucalyptus grandis, Boea hygrometrica, Physcomitrella patens and Populus trichocarpa between thirteen and eighteen ELIP genes are present, [36] [37], fewer are present in Rhododendron catawbiense, Oryza sativa, Fragaria vesca, Brassica rapa and Solanum lycopersicum, ranging from two to seven identified ELIP genes [38,39]. In the S. tuberosum genome only a single ELIP gene has been reported [40] as well as in the Musa acumunita, vitis, pea and tobacco genomes.

In plants and algae, isolated ELIPs show very unusual pigment-binding characteristics and pigment composition, with an extremely high lutein content when compared with other chlorophyll-binding proteins [41]. It is believed that these proteins fulfill a photo-protective role under light stress conditions. In saffron, all ELIP genes were induced by light but at very different levels. ELIPa showed the highest induction under light conditions followed by ELIPc. Both genes showed high expression levels in full-developed stigmas, and their expression was clearly increased in postanthesis. This last behavior is also followed by ELIPb. CsNCED, a gene encoding for a key enzyme involved in ABA biosynthesis in saffron [42], showed the highest levels of expression in stigmas at postanthesis, suggesting a correlation of ELIPa, b and c expression with stigma senescence, controlled in Crocus flowers by ABA. In fact, ELIPa and c expression levels increased by different abiotic stresses and ELIPc transcripts were clearly induced by ABA. In other plant species, ELIP genes have been shown to be up-regulated in responses to ABA [43], and in tobacco and pea it has been reported that ELIP was strongly up regulated during senescence [44,45].

Most of the studies on ELIP have been carried out in photosynthetic tissues, and much less is known about the role of ELIP in non-photosynthetic tissues. In tomato, ELIP has been suspected to play a role in chromoplastogenesis due to an increased expression during the transition from chloroplast to chromoplast, and its abundance in flowers containing yellow chloroplasts [39]. In citrus ELIP transcript levels are relatively low until the breaker stage, when the expression levels increase dramatically, before peaking at the ripe stage and declining thereafter. During this transition, the degradation of chlorophyll is concomitant with carotenoid biosynthesis and sequestration [46]. A carotene biosynthesis related gene (cbr) from a unicellular green algae Dunaliella bardawil, can be induced to accumulate massive amounts of β-carotene under light-stress conditions. Cbr was shown to accumulate coordinately with carotenogenesis induction, suggesting a function as pigment-binding protein, acting as a deposit of newly formed carotenoids [47,48]. These findings suggest the involvement of ELIP in carotenoid sequestration in plastoglobules and positively associated with carotenoid over-accumulation in chromoplasts as a mechanism of photoprotection.
The SOUL family in saffron

SOUL/HBP proteins were first detected in the retina and the pineal gland of chickens, suggesting a role in light signaling in vertebrates [34], but their presence in other tissues suggested additional roles including control of apoptosis, supply of oxygen to metabolically active tissues, or as oxygen sensors for transcription factors that respond to oxygen levels [35]. In green algae, SOUL proteins are associated to the regulation of the eyespot size and position [49], while in Arabidopsis, SOUL proteins are involved in phytochrome-mediated red/far-red light responses [50] and implicated to heme oxygenase-mediated antioxidant pathway [51]. Moreover, it has also been proposed that SOUL proteins in plants could participate in TSPO-dependent stress responses as soluble porphyrin transporters among the different compartments of the cell [52]. SOUL proteins are encoded by a multigene family in plants (https://phytozome.jgi.doe.gov) [53]. Arabidopsis contains six SOUL encoding genes, but one seems to be a pseudogene, and four are present in Chlamydomonas. Five members that belong to the SOUL family have been isolated in this study from saffron stigmas and each gene was clustered independently in different groups as well as with different predicted locations in the cell. The most unrelated group contains the saffron SOULE, predicted to be localized in plastids and mainly expressed in leaves. SOULE appears in the same group of Chlamydomonas SOUL3 and AtHBP5 heme proteins. Chlamydomonas SOUL3 has been the best studied SOUL protein in this organism, it was found in the eyespot proteome and phosphoproteome [54,55] and it was located in the pigment globule layer. SOUL3 influences in size and position of the eyespot in the cell [49]. Beside its function as a sensor for light direction and quality, the eyespot might have additional roles mainly in chloroplast function, such as the biosynthesis of prenylquinones and carotenoids [56]. It is hypothesized that the carotenoid-rich eyespot globule was originated from thylakoid membrane-associated PGs [56,57]. In the D. bardawil PG proteome, five distinct SOUL proteins have been isolated, including a homolog of Chlamydomonas SOUL3. In D. bardawil plastoglobuli several unique proteins have been identified including the major lipid-associated protein CGP [48,58]. The sequence of CGP reveals partial homology to SOUL heme-binding proteins and its relationship with the proteins of group 1. Proteolysis of CGP destabilizes the PGs, indicating that CGP may have a similar role to fibrillins acting as a stabilizer of the plastoglobules [59]. Further, Phylogenetic analyses showed the presence of an Arabidopsis homolog to SOUL3, AED92800, corresponding to AtHBP5, a chloroplast localized enzyme that participates in an antioxidant pathway that might be mediated by reaction products of heme catabolism. AtHBP5 may bind and then transport the excess heme to plastoglobules [51].

The second SOUL protein identified in saffron which was predicted to be localized in plastids, was SOULA. The SOULA homolog in Arabidopsis (ABG48434, At3g10130) has been found in the plastoglobule proteome [60]. This SOUL heme-binding protein of the plastoglobules is able to bind heme and is suggested to be involved in chlorophyll degradation processes [61]. However, SOULA was mainly expressed in stigmas, where no chloroplasts are present, and was developmentally regulated, suggesting a different role for SOULA in saffron which has no relationship with chlorophyll degradation. Further, the expression of SOULA is associated with the increased number of plastoglobules in saffron chromoplasts [6], and its expression was induced by several stresses, including drought and desiccation. Increased numbers of PGs have been related to various stress factors such as excess of light, drought, senescence, desiccation, and nutrient deficiency [34]. PGs in chromoplasts contain mostly carotenoids and enzymes responsible for carotenoid biosynthesis and metabolism but also other enzymes of unknown function [60]. Among the carotenogenic enzymes, heme serves as a prosthetic group in the Z-ISO isomerase [62] and in the carotenoid ε hydroxylases that catalyze hydroxylation
of the epsilon-ring of beta, epsilon-carotenoids [63]. It is possible that SOUL proteins may transport heme to the chromoplast and to the PGs to maintain heme homeostasis.

Saffron SOULb was mainly expressed in stigmas at anthesis and in tepals, and was the unique SOUL gene from saffron to be induced by light in stigmas. The chlamydomonas homolog SOUL2 was identified in a recent work from the proteomic analyses of the intact eyespot of Chlamydomonas [64], although its role has not been determined. The eyespot located in the chloroplast is composed of photoreceptor proteins and red to orange carotenoid pigments, allowing the detection of light directionality. A SOULb homologue has also been identified in tomato, and was differentially expressed during fruit maturation, with higher levels of expression in the ripe stage [65].

SOULc from saffron showed in general the lower expression levels among the other SOUL genes, with the highest expression in the red stigma. Interestingly, AtHBP2 (AEC09472, At2g37970), the SOULc homolog in Arabidopsis, was initially identified as a phytochrome A-induced transcript that rapidly responded to light during de-etiolation [50]. However, SOULc was not induced by light under our experimental conditions. Takahashi et al. [66] showed that AtHBP2 bound porphyrins, including heme, and they proposed that it was a tetrapyrrole-carrier protein. This protein is supposed to be in the cytosol where it can transport heme among cellular organelles. The same function could be suggested for SOULd. The expression of SOULd was mainly detected in saffron stigmas, and its expression increased during the development of the stigma, reaching the highest levels after anthesis, but was not induced by light. SOULd is predicted to be involved in the secretory pathway, as its Arabidopsis homolog AtHBP1. In pepper a SOULd homolog was identified as a secreted protein in fruit ripening [67]. In addition, the xSOUL protein from Xenopus, containing a cleavable signal peptide, was detected as a secreted protein, and suggested to be involved in cell-cell communication [68], or might function on the cell surface as a light or redox sensor by means of the heme ligand.

A link between ELIP and SOUL through HY5

Light is a key factor controlling plant development and acclimation processes, by regulating growth and adapting to environmental conditions plant organelles and the nucleus communicate with each other. Plastid-to-nucleus communication is of particular importance during plant stress responses associated with light. Under our research conditions, SOUL, ELIP and HYH1 (HY5-homolog) and HY5 homologues were identified as the highest induced genes by light in saffron stigmas. HY5 is an important positive regulator of light-dependent gene expression that is regulated by multiple photoreceptors. HY5 is necessary for the rapid transcription of genes during the dark-to-light transition [11] and could act as a signal transducer that links hormone and light signals [69]. Numerous studies have been shown that HY5 responds to an unknown plastid signal and is suggested to be involved in retrograde signaling pathways demonstrating a convergence between plastid and light signaling networks [70]. In Arabidopsis, HY5 promotes the light induction of Elip1 [33] and Elip2 [71], and the AtHBP2 promoter was identified as a target of HY5 [11]. Such findings suggest that in saffron ELIPA and SOULb could be targets of HY5 when stigmas pass from dark to light conditions.

Several ELIP and SOUL isoforms have been detected among the proteins from PGs proteome, in different cases a structural function has been suggested as associated to the integrity of PGs under high-light conditions. In fact, the regulation of ELIP and SOUL expression is modulated by light and other stress signals, but is also developmentally regulated. Photo-oxidative damage must be a primary stressor for Crocus species, as they spend a considerable amount of time below ground and when they emerge they must cope with high-light conditions. Thus, it appears that saffron has evolved a strategy of ELIP gene expansion to aid in its ability to protect
its chromoplasts from oxidative damage. In addition, abiotic stress responses generically involve the production of reactive oxygen species (ROS) in plant cells, and heme plays important roles in ROS detoxification. SOUL proteins may act as carriers to balance and buffer the state of free heme in the plastid.

**Materials and Methods**

**Plant materials**

Plant tissues and stigmas at different developmental stages were obtained from *C. sativus* grown under field conditions in the Botanical Garden of CLM (Albacete, Spain). The tissues were frozen in liquid nitrogen and stored at -80˚C until required. For stress-induced treatments, red stigmas were transferred for 6 hrs. to 24-well-plates containing 1 ml water supplemented with 100 mM NaCl or distilled water that was used as a control; and incubated under simulated field conditions (12 h light/dark cycles at 22˚C/10˚C). Heat stress was applied by raising the temperature to 38˚C for 6 h. For the dehydration experiments the stigmas were incubated under simulated field conditions in Petri dishes on Whatman No. 3MM paper (Whatman International, Maidstone, England) and collected 24 hours later.

**Construction of the subtracted cDNA libraries**

For cDNA library construction, saffron corms growing in the field were transferred during one week to growth chambers simulating field conditions (12 h light/dark cycles at 22˚C/10˚C). 24 h before the collection of the material, the light in one chamber was constant and in the other chamber the samples were incubated under no light. Flowers were dissected and red stigmas were used in the experiment. Total RNA was isolated from dark (control) and light-treated red stigmas using the RNeasy Plant Mini Kit (Qiagen, Germany). For PCR-select cDNA subtraction, mRNAs were purified with the Oligotex™ mRNA Mini Kit (Qiagen, Germany). The suppression subtractive hybridization (SSH) cDNA libraries, forward (light-treated) and reverse (dark control), were prepared using a PCR-Select cDNA Subtraction Kit (Clontech, California, USA) and following the manufacturer’s instructions. Two rounds of hybridization and PCR amplification were performed to normalize and enrich differentially expressed cDNA. The subtracted cDNA was further cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into chemically competent Stellar Escherichia coli cells (Clontech, Takara, Japan). The transformed cells were selected on LB agar plates containing ampicillin (100 μg/mL) at 37˚C o/n for screening.

**Identification of cDNA inserts sizes by PCR**

300 white clones were randomly selected from the plates and then incubated in liquid medium supplemented with ampicillin overnight at 37˚C. The SSH cDNA clone inserts were amplified by PCR using M13F and M13R primers by 20 cycles of 94˚C for 30 s, 52˚C for 30 s, and 72˚C for 1.5 min. A 5-μL aliquot of each PCR product was electrophoresed on 1% agarose gels. The rest of the PCR reactions were used for screening by dot-blot hybridization. Briefly, the PCR product (10 μL) was denatured by adding 1 M NaOH and 200 mM EDTA, pH 8.2, to give a final concentration of 0.4 M NaOH/10 mM EDTA. Samples were heated for 10 min at 100˚C and spotted onto two identical nylon positively charged membranes (GE Healthcare Amer sham™). The two blots were UV cross-linked and hybridized with DIG-labeled forward- and reverse-subtracted cDNA probes that were prepared using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Hybrids were detected with alkaline phosphatase-conjugated antibody against DIG (1:1000 dilution of anti-DIG-AP). The signals were generated by
treating membranes with 1% CSPD, and exposed to an X-ray film (Fuji Biomax MR film) for varying times (e.g. 1, 6, 24 and 48 h).

DNA sequencing and data analysis

Plasmids were sequenced using an automated DNA sequencer (ABI PRISM 3730xL, Perkin Elmer, Macrogen Inc., Seoul, Korea). Computer-aided sequence similarity searches were made with the BLAST suite of programs from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Motif searches were made using SignalP (http://www.cbs.dtu.dk/services/SignalP) and TMPRED (http://www.isrec.isb-sib.ch/software/software.html). The proteins were modeled using the phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/) and the PPM server (http://opm.phar.umich.edu/server.php). ELIP proteins were modeled based on c4ri3A, the crystal structure of dccd-modified psbs from spinach. SOUL proteins were modeled based on d2gova1.

Validation of SSH analysis by quantitative PCR

We employed quantitative PCR to verify the differences in gene expression of selected target genes identified by SSH. Primers designed for each gene are given in S1 Table. Quantitative PCR was done with a StepOne™ Thermal Cycler (Applied Biosystems, Foster City, California, USA) and analyzed using StepOne software v2.0 (Applied Biosystems, Foster City, California, USA). Gene changes were determined using the 2^−ΔΔCt method by normalizing to the 18S rRNA (first Δ) and then the expression was calculated for each gene by comparison to the normalized expression registered for dark conditions (second Δ).

Phylogenetic analysis

The amino acid sequences were aligned using the BLOSUM62 matrix with the ClustalW (http://www.clustal.org) algorithm-based AlignX module from MEGA Version 6.0 (Tamura et al., 2013) (http://www.megasoftware.net/mega.html). The alignments were saved and executed by MEGA Version 6.0 to generate a Neighbour Joining Tree with bootstrapping (2000 replicates) analysis and handling gaps with pairwise deletion.

Gene expression by quantitative reverse transcription-PCR (qRT-PCR)

Gene-specific oligonucleotides were used for the expression analyses (S1 Table). Total RNAs were isolated from C. sativus stigmas at seven developmental stages (white, yellow, orange, red, preanthesis, anthesis and postanthesis stigma) and from tepals, stamens, leaves, roots, corms and cormlets, by grinding fresh tissue in liquid nitrogen to a fine powder and extracting in 1 ml of Trizol reagent (Gibco-BRL) per 100 mg of fresh tissue weight, according to the manufacturer’s protocol. The RNA obtained was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). First-strand cDNAs were synthesized by RT from 1–2 μg of total RNA using a first-strand cDNA synthesis kit from GE Healthcare Life Sciences (Buckinghamshire, UK) and 18mer oligo dT. The quantitative RT-PCR was carried out on cDNA from three biological replicates; reactions were set up in a final volume of 25 μl in GoTaq® qPCR Master Mix (Promega, Madison, WI, USA) according to manufacturer’s instructions. The constitutively expressed 18S rRNA gene was used as a reference gene [72]. The qPCR conditions consisted in an initial denaturation at 94˚C for 5 min; followed by 40 subsequent cycles of denaturation at 94˚C for 20 s, annealing at 58˚C for 20 s and extension at 72˚C for 20 s; and finally extension at 72˚C for 5 min. The assays were conducted with a StepOne™ Thermal Cycler (Applied Biosystems, Foster City, California, USA) and analyzed using StepOne software v2.0 (Applied
Biosystems, Foster City, California, USA). DNA melt curves were created for each primer combination to confirm the presence of a single product.

Supporting Information

S1 Fig. (PPTX)

S1 Table. (DOCX)

Acknowledgments

We thank K.A. Walsh for language revision. This work was supported by the Spanish Ministerio de Economía y Competitividad (BIO2013-44239-R). The laboratory participates in the CARNET network (BIO2015-71703-REDT). The authors declare that they have no conflict of interest.

Author Contributions

Conceptualization: OA LGG.

Formal analysis: OA AR LGG.

Funding acquisition: LGG.

Investigation: OA JA RC AR LGG.

Methodology: OA AR LGG.

Project administration: LGG.

Resources: LGG.

Supervision: OA LGG.

Validation: OA JA LGG.

Visualization: OA AR RC JA LGG.

Writing – original draft: OA LGG.

Writing – review & editing: OA JA AR LGG.

References

1. Moraga AR, Rambla JL, Ahrazem O, Granell A, Gomez-Gomez L (2009) Metabolite and target transcript analyses during Crocus sativus stigma development. Phytochemistry 70: 1009–1016. doi: 10.1016/j.phytochem.2009.04.022 PMID: 19473679

2. Ahrazem O, Rubio-Moraga A, Lopez RC, Gomez-Gomez L (2010) The expression of a chromoplast-specific lycopene beta cyclase gene is involved in the high production of saffron's apocarotenoid precursors. J Exp Bot 61: 105–119. doi: 10.1093/jxb/erp283 PMID: 19767307

3. Castillo R, Fernandez JA, Gomez-Gomez L (2005) Implications of carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of Crocus sativus and its closer relatives. Plant Physiol 139: 674–689. doi: 10.1104/pp.105.067827 PMID: 16183835

4. Ahrazem O, Rubio-Moraga A, Argandona-Picazo J, Castillo R, Gomez-Gomez L (2016) Intron retention and rhythmic diel pattern regulation of carotenoid cleavage dioxygenase 2 during crocetin biosynthesis in saffron. Plant Mol Biol.
5. Ahrazem O, Rubio-Moraga A, Nebauer SG, Molina RV, Gomez-Gomez L (2015) Saffron: Its Phytochemistry, Developmental Processes, and Biotechnological Prospects. J Agric Food Chem 63: 8751–8764. doi: 10.1021/acs.jafc.5b03194 PMID: 26414550
6. Grilli Caiola M, Canini A (2004) Ultrastructure of chloroplast and other plastids in Crocus sativus L. (Iridaceae). Plant Biosystems 138: 9.
7. Rubio A, Rambla JL, Santaela M, Gomez MD, Orzaez D, et al. (2008) Cytosolic and plastoglobule-targeted carotenoid dioxygenases from Crocus sativus are both involved in beta-ionone release. J Biol Chem 283: 24816–24825. doi: 10.1074/jbc.M804000200 PMID: 18611853
8. Ruiz-Sola MA, Rodriguez-Concepcion M (2012) Carotenoid biosynthesis in Arabidopsis: a colorful pathway. Arabidopsis Book 10: e0158. doi: 10.1199/tab.0158 PMID: 22582030
9. Llorente B, D’Andrea L, Ruiz-Sola MA, Botterweg E, Pulido P, et al. (2016) Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. Plant J 85: 107–119. doi: 10.1111/pj.13094 PMID: 26648446
10. Llorente B, D’Andrea L, Rodriguez-Concepcion M (2016) Evolutionary Recycling of Light Signaling Components in Fleshy Fruits: New Insights on the Role of Pigments to Monitor Ripening. Front Plant Sci 7: 263. doi: 10.3389/fpls.2016.00263 PMID: 27014289
11. Lee J, He K, Stolc V, Lee H, Figueroa P, et al. (2007) Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell 19: 731–749. doi: 10.1105/tpc.106.047688 PMID: 17337630
12. Liu L, Shao Z, Zhang M, Wang Q (2015) Regulation of carotenoid metabolism in tomato. Mol Plant 8: 28–39. doi: 10.1016/j.molp.2014.11.006 PMID: 25578270
13. Cheung AY, McNellis T, Piekos B (1993) Maintenance of Chloroplast Components during Chromoplast Differentiation in the Tomato Mutant Green Flesh. Plant Physiol 101: 1223–1229. PMID: 8650213
14. Lagod J, Cronje P, Alquezar B, Page A, Manzi M, et al. (2015) Fruit shading enhances peel color, carotenes accumulation and chromoplast differentiation in red grapefruit. Physiol Plant 154: 469–484. doi: 10.1111/plp.12332 PMID: 25676857
15. McCollum JP (1954) Effects of light on the formation of carotenoids in tomato fruits. Journal of Food Science 19: 7.
16. Champagne A, Bernillon S, Moing A, Rolin D, Legendre L, et al. (2010) Carotenoid profiling of tropical root crop chemotypes from Vanuatu, South Pacific. J Food Comp Anal 23: 8.
17. Rodriguez-Concepcion M, Stange C (2013) Biosynthesis of carotenoids in carrot: an undergrou nd story comes to light. Arch Biochem Biophys 539: 110–116. doi: 10.1016/j.abb.2013.07.009 PMID: 23876238
18. Ferreira CF, Alves E, Pestana KN, Junghans DT, Kobayashi AK, et al. (2008) Molecular characterization of cassava (Manihot esculenta Crantz) with yellow-orange roots for beta-carotene improvement. Crop Breed Appl Biotechnol 8: 6.
19. Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, et al. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 93: 6025–6030. PMID: 8690210
20. Noordally ZB, Ishii K, Atkins KA, Wetherill SJ, Kusakina J, et al. (2013) Circadian control of chloroplast transcription by a nuclear-encoded timing signal. Science 339: 1316–1319. doi: 10.1126/science.1230397 PMID: 24349371
21. Hardtke CS, Gohda K, Osterlund MT, Oyama T, Okada K, et al. (2000) HY5 stability and activity in Arabidopsis. EMBO J 19: 4997–5006. doi: 10.1093/emboj/19.18.4997 PMID: 10990463
22. Holm M, Ma LG, Qu LJ, Deng XW (2002) Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in Arabidopsis. Genes Dev 16: 1247–1259. doi: 10.1101/gad.969702 PMID: 12023303
23. Grimm B, Kloppstech K (1987) The early light-inducible proteins of barley. Characterization of two families of 2-h-specific nuclear-coded chloroplast proteins. Eur J Biochem 167: 493–499. PMID: 2443530
24. Zylka MJ, Reppert SM (1999) Discovery of a putative heme-binding protein family (SOUL/HBP) by two-tissue suppression subtractive hybridization and database searches. Brain Res Mol Brain Res 74: 175–181. PMID: 10640688
25. Adamska I, Kruse E, Kloppstech K (2001) Stable insertion of the early light-induced proteins into etioplast membranes requires chlorophyll a. J Biol Chem 276: 8582–8587. doi: 10.1074/jbc.M010447200 PMID: 11114311
26. Heddad N, Noren H, Reiser V, Dunaeva M, Andersson B, et al. (2006) Differential expression and localization of early light-induced proteins in Arabidopsis. Plant Physiol 142: 75–87. doi: 10.1104/pp.106.081489 PMID: 16829598
27. Green BR, Kuhlbrandt W (1995) Sequence conservation of light-harvesting and stress-response proteins in relation to the three-dimensional molecular structure of LHCII. Photosynth Res 44: 139–148. doi: 10.1007/BF00018304 PMID: 24307033
28. Kuhlbrandt W, Wang DN, Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621. doi: 10.1038/367614a0 PMID: 8107845
29. Wang H, Cao F, Li G, Yu W, Aitken SN (2015) The transcript profiles of a putative early light-induced protein (ELIP) encoding gene in Ginkgo biloba L. under various stress conditions. Acta Physiologica Plantarum 37.
30. Berti M, Pinto M (2012) Expression of Early Light Induced Protein in Grapevine and Pea, under Different Conditions and its Relation with Photoinhibition. Chilean journal of agricultural research 72: 371–378.
31. Ambrosi E, Capaldi S, Bovi M, Saccomani G, Perduca M, et al. (2011) Structural changes in the BH3 domain of SOUL protein upon interaction with the anti-apoptotic protein Bcl-xL. Biochem J 438: 291–301. doi: 10.1042/BJ20110257 PMID: 21639858
32. Doczi R, Brader G, Pettko-Szandtner A, Rajh I, Djamei A, et al. (2007) The Arabidopsis mitogen-activated protein kinase kinase MKK3 is upstream of group C mitogen-activated protein kinases and participates in signaling pathways. Plant Cell 19: 3266–3279. doi: 10.1105/tpc.106.050039 PMID: 17933903
33. Harari-Steinberg O, Ohad I, Chamovitz DA (2001) Dissection of the light signal transduction pathways regulating the two early light-induced protein genes in Arabidopsis. Plant Physiol 127: 986–997. PMID: 11706180
34. Xu X, Pan S, Cheng S, Zhang B, Mu D, et al. (2011) Genome sequence and analysis of the tuber crop potato. Nature 475: 189–195. doi: 10.1038/nature10158 PMID: 21743474
35. Adamska I, Roobol-Boza M, Lindahl M, Andersson B (1999) Isolation of pigment-binding early light-inducible proteins from pea. Eur J Biochem 260: 453–460. PMID: 10095781
36. Ahrazem O, Rubio-Moraga A, Trapero A, Gomez-Gomez L (2012) Developmental and stress regulation of gene expression for a 9-cis-epoxycarotenoid dioxygenase, CstNCE D, isolated from Crocus sativus stigmas. J Exp Bot 63: 681–694. doi: 10.1093/jxb/erq293 PMID: 22048040
37. Zeng Q, Chen X, Wood AJ (2002) Two early light-inducible protein (ELIP) cDNAs from the resurrection plant Tortula ruralis are differentially expressed in response to desiccation, rehydration, salinity, and high light. J Exp Bot 53: 1197–1205. PMID: 11971930
38. Binyamin L, Falah M, Portnoy V, Soudry E, Gepstein S (2001) The early light-induced protein is also produced during leaf senescence of Nicotiana tabacum. Planta 212: 591–597. doi: 10.1007/s004250000423 PMID: 11525516
39. Norén H, Svensson P, Stegmark R, Funk C, Adamska I, et al. (2003) Expression of the early light-induced protein but not the PsbS protein is influenced by low temperature and depends on the developmental stage of the plant in field-grown pea cultivars. Plant, Cell & Environment 26: 245–253.
40. Alos E, Roca M, Iglesias DJ, Minguez-Mosquera MI, Damasceno CM, et al. (2008) An evaluation of the basis and consequences of a stay-green mutation in the navel negra citrus mutant using transcriptomic and proteomic profiling and metabolite analysis. Plant Physiol 147: 1300–1315. doi: 10.1104/pp.108.119917 PMID: 18467459
41. Lers A, Levy H, Zamir A (1991) Co-regulation of a gene homologous to early light-induced genes in higher plants and beta-carotene biosynthesis in the alga Dunaliella bardawil. J Biol Chem 266: 13698–13705. PMID: 1856203
48. Katz A, Jimenez C, Pick U (1995) Isolation and Characterization of a Protein Associated with Carotene Globules in the Alga Dunaliella bardawil. Plant Physiol 108: 1657–1664. PMID: 12228570

49. Schulze T, Schreiber S, Iliiev D, Boesger J, Trippens J, et al. (2013) The heme-binding protein SOUL3 of Chlamydomonas reinhardtii influences size and position of the eyespot. Mol Plant 6: 931–944. doi: 10.1093/mp/ssr017 PMID: 23180671

50. Khanna R, Shen Y, Toledo-Ortiz G, Kikis EA, Johannesson H, et al. (2006) Functional profiling reveals that only a small number of phytochrome-regulated early-response genes in Arabidopsis are necessary for optimal deetiolation. Plant Cell 18: 2157–2171. doi: 10.1105/tpc.106.042200 PMID: 16891401

51. Lee HJ, Mochizuki N, Masuda T, Buckhouit TJ (2012) Disrupting the bimolecular binding of the haem-binding protein 5 (AtHBP5) to haem oxygenase 1 (HY1) leads to oxidative stress in Arabidopsis. J Exp Bot 63: 5967–5978. doi: 10.1093/jxb/ers242 PMID: 22991161

52. Vanhee C, Batoko H (2011) Arabidopsis TSPO and porphyrins metabolism: a transient signaling connection? Plant Signal Behav 6: 383–385. doi: 10.4161/psb.6.9.16477 PMID: 21852759

53. Fortunato AE, Sordino P, Andreakis N (2016) Evolution of the SOUL Hemebinding Protein Superfamily Across Eukarya. J Mol Evol 82: 279–290. doi: 10.1007/s00239-016-0745-9 PMID: 27209522

54. Vagner V, Ullmann K, Mollwo A, Kaminski M, Mitiag M, et al. (2008) The phosphoproteome of a Chlamydomonas reinhardtii eyespot fraction includes key proteins of the light signaling pathway. Plant Physiol 146: 772–788. doi: 10.1104/pp.107.109645 PMID: 18065559

55. Kreimer G (2009) The green algal eyespot apparatus: a primordial visual system and more? Curr Genet 55: 19–43. doi: 10.1007/s00294-008-0224-8 PMID: 19107486

56. Boyd JS, Mittelmeier TM, Dieckmann CL (2011) New insights into eyespot placement and assembly in Chlamydomonas. Bioarchitecture 1: 196–199. doi: 10.4161/bioa.1.4.17697 PMID: 22069514

57. Davidi L, Levin Y, Ben-Dor S, Pick U (2015) Proteome analysis of cytoplasmic beta-carotene lipid droplets in Dunaliella bardawil. Plant Physiol 167: 60–79. doi: 10.1104/pp.114.248450 PMID: 25404729

58. Singh DK, McNellis TW (2011) Fibrillin protein function: the tip of the iceberg? Trends Plant Sci 16: 432–441. doi: 10.1016/j.tplants.2011.03.014 PMID: 21571574

59. Ytterberg AJ, Peltier JB, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chro- moplasts. A surprising site for differential accumulation of metabolic enzymes. Plant Physiol 140: 984–997. doi: 10.1104/pp.105.076083 PMID: 16461379

60. Lundquist PK, Poliakov A, Bhuiyan NH, Zybailov B, Sun Q, et al. (2012) The functional network of the Arabidopsis plastoglobule proteome based on quantitative proteomics and genome-wide coexpression analysis. Plant Physiol 158: 1172–1192. doi: 10.1104/pp.111.193144 PMID: 22274653

61. Beltran J, Kloss B, Hosler JP, Geng J, Liu A, et al. (2015) Control of carotenoid biosynthesis through a heme-based cis-trans isomerase. Nat Chem Biol 11: 596–605. doi: 10.1038/nchembio.1840 PMID: 26075523

62. Tian L, DellaPenna D (2004) Progress in understanding the origin and functions of carotenoid hydroxylases in plants. Arch Biochem Biophys 430: 22–29. doi: 10.1016/j.jbabio.2004.02.003 PMID: 15325908

63. Eitzinger N, Wagner V, Weishart W, Geimer S, Boness D, et al. (2015) Proteomic Analysis of a Fraction with Intact Eyespots of Chlamydomonas reinhardtii and Assignment of Protein Methylation. Front Plant Sci 6: 1085. doi: 10.3389/fpls.2015.01085 PMID: 26697039

64. Page D, Marty I, Bouchet JP, Gouble B, Causse M (2008) Isolation of genes potentially related to fruit quality by subtractive selective hybridization in tomato. Postharvest Biology and Technology 50: 7.

65. Takahashi S, Ogawa T, Inoue K, Masuda T (2008) Characterization of cytosolic tetrapyrrole-binding proteins in Arabidopsis thaliana. Plant Physiol Biochem 46: 6.

66. Lee JM, Lee S-J, Rose JKC, Yeam I, Kim BD (2014) Mining secreted proteins that function in pepper fruit development and ripening using a yeast secretion trap (YST). Biochemical and Biophysical Research Communications 446: 6.

67. Pera EM, Hou S, Strate I, Wessely O, De Robertis EM (2005) Exploration of the extracellular space by a large-scale secretion screen in the early Xenopus embryo. Int J Dev Biol 49: 781–796. doi: 10.1387/ijdb.052003ep PMID: 16172975

68. Cluis CP, Mouchel CF, Hardtke CS (2004) The Arabidopsis transcription factor HYS integrates light and hormone signaling pathways. Plant J 38: 332–347. doi: 10.1111/j.1365-313X.2004.02052.x PMID: 15078335
70. Waters MT, Wang P, Korkaric M, Capper RG, Saunders NJ, et al. (2009) GLK transcription factors coordinate expression of the photosynthetic apparatus in Arabidopsis. Plant Cell 21: 1109–1128. doi: 10.1105/tpc.108.065250 PMID: 19376934

71. Hayami N, Sakai Y, Kimura M, Saito T, Tokizawa M, et al. (2015) The Responses of Arabidopsis Early Light-Induced Protein2 to Ultraviolet B, High Light, and Cold Stress Are Regulated by a Transcriptional Regulatory Unit Composed of Two Elements. Plant Physiol 169: 840–855. doi: 10.1104/pp.15.00398 PMID: 26175515

72. Rubio-Moraga A, Trapero A, Ahrazem O, Gomez-Gomez L (2010) Crocins transport in Crocus sativus: the long road from a senescent stigma to a newborn corm. Phytochemistry 71: 1506–1513. doi: 10.1016/j.phytochem.2010.05.026 PMID: 20573363