Surveying the Lettuce Genome: Genome-wide Identification of Hsfs and Hsps in Response to UV and High Light Stress

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Working title: Identification of Hsfs and Hsps in lettuce

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Abstract

Background

Heat shock factors (Hsfs) and Heat shock proteins (Hsps) belong to an essential group of molecular regulators involved in controlling cellular processes under normal and stress conditions. The role of Hsfs and Hsps is well known in model plant species under diverse stress conditions. However, in lettuce (*Lactuca sativa*), a highly consumed vegetable crop grown in the field and in hydroponic systems, the role of these gene families in response to artificial light is not well characterized.

Results

Using a genome-wide analysis approach, we identified 32 Hsfs and 22 small heat shock proteins (LsHsps) in lettuce, some of which do not have orthologous in Arabidopsis, poplar, and rice. LsHsp60s, LsHsp90s, and LsHsp100s are highly conserved among dicot and monocot species. Surprisingly, LsHsp70s have three times more members than Arabidopsis and two times more than rice. Genome triplication might have contributed to the increased number of LsHsp70s genes. Chromosomal distribution analysis shows larger tandem repeats of LsHsp70s genes in Chr1, Chr7, Chr8, and Chr9. At the transcriptional level, some genes of the LsHsfs, LsHsps, LsHsp60s, and LsHsp70s families were highly responsive to UV and high light stress, in contrast of LsHsp90s and LsHsp100s which did not respond to a light stimulus.

Conclusions

Our genome-wide analysis provides a detail identification of Hsfs and Hsps in lettuce. Chromosomal location and syntenic region analysis together with our transcriptional analysis under different light conditions provide candidate genes for breeding programs aiming to

produce lettuce varieties able to grow healthy under hydroponic systems that use artificial light.

Background

Increasing human population, climate change conditions, decrease in water availability, and pathogens and insect pressure have influenced the way we grow crops. Hydroponics is a viable alternative of production to address many of these issues [1]. Vegetables and fruits are the most commonly grown hydroponic crops. Within these group, lettuce (*Lactuca sativa*) is one of the most common vegetables produced in hydroponic systems [1–3]. Some of the production and health benefits of lettuce include a short production cycle, small size as well as its rich fiber, vitamins, minerals, and phytochemicals content [4].

Lettuce is an important recently sequenced vegetable crop with a diploid genome ($2n=2x=18$ chromosomes) and an estimated size of 2.5 Gb [5]. A large fraction of lettuce production is grown hydroponically and indoors, relying on the supply of a nutrient solution and artificial light. The intensity and properties of light are critical factors that regulate photosynthesis and plant growth. Optimization of nutrients and light throughout the production cycle can provide better growing conditions to indoor hydroponic systems [2, 3]. However, the constant supply of high-energy radiation in the form of UV or high light intensity can negatively alter not only plant growth and development [6, 7], but also the transcriptional pattern of gene expression [8, 9]. Under these conditions, the expression of stress responsive genes is critical to ensure plant fitness and normal development. Two gene families are known to be in the front line of stress

responses to tackle adverse conditions, heat shock factors (Hsfs) and heat shock proteins (Hsps). Hsfs and Hsps are involved in stress response mechanisms that allow plants to control folding, accumulation, and degradation of proteins.

Heat shock factors are transcriptional activators that regulate gene expression of their target genes. In plants, Hsfs are classified into three groups, Hsfs type A, B, and C. Type A is the largest group of Hsfs in most species including *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) [10–12]. Type B contains five genes and type C is the less diverse and contain only a few gene members. In contrast, Hsps are more abundant and are classified based on their molecular weight into five subfamilies, small Hsps (sHsps), Hsp60s, Hsp70s, Hsp90s, and Hsp100s. Members of Hsfs and Hsps have been described in response to heat, cold, drought, and salinity in many monocots and dicots species [10–14].

Most studies investigating lettuce in response to light have been focused on the effects of high-energy radiation with ultraviolet (UV) and high light intensity wavebands on crop quality [2] and yield [3]. However, the molecular responses of Hsfs and Hsps to different light treatments have not been characterized. Here, we describe a genome-wide analysis of Hsfs and Hsps genes under diverse light conditions. A comprehensive description of subclasses of Hsfs, sHsps, Hsp60s, Hsp70s and Hsp100s has been generated providing novel candidate genes for lettuce breeding programs.

Results

Identification and properties of Lettuce Heat Shock Transcription Factors

Heat shock transcription factors (Hsfs) are important transcriptional activators of stress-regulated genes. Based on the recent sequencing of the lettuce genome [5], we identified LsHsfs family members using an automated local BLASTP search with the previously described Arabidopsis [10], rice [11], and poplar Hsfs [12] as query sequences followed by manual curation. Previous Hsfs genome-wide studies have identified 21 AtHsfs, 28 OsHsfs, and 25 PtHsfs. Our automated search yielded 32 members of the LsHsfs family (Fig. 1A). Considering that the sequenced lettuce genome is a diploid plant with an estimated genome size of 2.5 Gb, it is interesting to note that lettuce carries a higher number of Hsfs compared to rice and poplar.

To further characterize LsHsfs, we collected their physiochemical features including chromosomal coordinates, molecular weight (MW), theoretical isoelectric point (pI), instability index, aliphatic index, hydropathicity, and predicted subcellular localization (Additional file 1: Table S1). Members of the LsHsfs family showed largely variable MWs, ranging from approximately 13 kDa to 54 kDa (Fig. 1B). Consistent with their putative function as transcription factors, most of the LsHsfs were predicted to be localized in the nucleus; however, LsHsfA1b and LsHsfA8 showed chloroplast signal peptides (Fig. 1B; Additional file 1: Table S1). It will be interesting to investigate whether both genes could play a dual function in the nucleus and the chloroplast. Another well conserved genome feature was the number of exons; LsHsfs family members have between 2 to 5 exons (Fig. 1C).

Because our analyses showed an increase in LsHsfs number, we decided to quantify the ratio of substitution rates at non-synonymous and synonymous sites in both LsHsfs and LsHsp to explore the evolutionary pressures on proteins during lettuce whole-genome triplication [5]. The largest dN/dS ratio was observed in LsHsfs (0.1); however, the hallmark signature of positive selection is accepted to be dN/dS>1 [15]. All the LsHsps families showed dN/dS ratio of 0.5 or below (Fig. 1D). In general, no evidence of positive selection was found on any other member of the LsHsfs and the different LsHsps families based on the dN/dS ratio analysis (Fig. 1D; Additional file 2: Table S2).

Classification, gene structure, and phylogenetic analysis of lettuce Hsfs

Since Arabidopsis Hsfs have been well characterized, we used their nomenclature to classify lettuce Hsfs. As the criteria, we selected sequences with an E-value threshold of $1E^{-20}$. LsHsfs were classified in three main groups (Fig. 2A); type A (13 genes), type B (12 genes) and type C (7 genes). To further assess the evolutionary relationship of the Hsfs, a phylogenetic tree was constructed based on the full-length amino acid sequences from both lettuce and Arabidopsis (Fig. 2B). While most members of the type A subfamily in Arabidopsis had a similar number of orthologous lettuce genes, we found only two members (LsHsfA1a and LsHsfA1b) orthologous with four Arabidopsis Hsf genes in the subgroup A1. LsHsfs Type B and C subfamilies showed a larger number of new members compared to Arabidopsis. Interestingly, we identified a new lettuce subgroup that is not present in Arabidopsis, that we named LsHsfB5. It has three members with high homology among them. Remarkably, we also identified a large number of

LsHsf Type C (seven genes) compared to Arabidopsis (one gene), which mainly contributed to the overall increase in number of the LsHsfs family (Fig. 2B). The gene structure of the LsHsfs was highly conserved; however, four members of the LsHsfs type C (LsHsfC1a, LsHsfC1b, LsHsfC1d, LsHsfC1e) and three of the LsHsfs type B (LsHsfB1a, LsHsfB1b, LsHsfB1c) family displayed different gene structures and variable motif distributions (Fig. 2C).

Lettuce Small Heat Shock Proteins (LssHsps)

We identified and classified lettuce small heat shock proteins (LssHsps) into nine subfamilies (Fig. 3A). Each subfamily was named based on their predicted MW and subcellular localization (Fig. 1B). Physiochemical features and predicted subcellular localization were also characterized (Additional file 1: Table S1). Five lettuce sHsp subfamilies are cytosol-localized (C-I, C-II, C-III, C-IV, and C-V). The other four subfamilies are predicted to be expressed in the endoplasmic reticulum (ER), peroxisome (PX), chloroplast (CP), and mitochondria (MT) (Fig. 3A-B). We found a large number of members belonging to the C-I (12 genes) and C-II subfamilies (6 genes) in the lettuce genome compared to only six C-I and two C-II genes in the Arabidopsis genome (Fig. 3B). Interestingly, we did not find LsHsp-CVI members based on their homology with Arabidopsis (Fig. 3B). Remarkably, the large majority of LssHsps have a single exon (Fig. 3C); nonetheless, the members of the LssHsp-PX, LssHsp-P, and LssHsp-M subclades have two or three exons. At the gene structure level, the exon/intron structures of LssHsps were relatively uniform except for LsHsp23.8-CI and LsHsp37.6-ER which contain long introns spanning 7.77 kb and 9.16 kb, respectively (Fig. 2C). In addition, each subfamily of LssHsps has its unique number of conserved motifs. Remarkably, LssHsps subfamilies predicted to be expressed in the cytosol

showed distinctive motifs (Motif 4) which could be an indication of their potential function in the plant cell (Fig. 3C).

LsHsp60, a well conserved gene family

Based on the phylogenetic relationships of the lettuce Hsp60 family with Arabidopsis Hsp60s, we classified LsHsp60 family into four subclasses: Cpn60 which contains 12 cytoplasmic genes, Cpn60 α having four chloroplastic members, Cpn60 β containing three chloroplastic members, and the Hsp60 subclass consisting of three mitochondrial genes (Fig. 4A). In general, Hsp60 genes are well conserved between Arabidopsis and lettuce given that each Arabidopsis Hsp60 member has at least one orthologous gene in lettuce. In addition, there are no Hsp60 lettuce-specific clade (Fig. 4B). At the gene structure level, the LsHsp60 has a well conserved motif structure; however, the number of exons varies across the different subfamilies (Fig. 4C). A physiochemical characterization of LsHsp60 was also conducted and can be found in Additional file 1: Table S1.

Another interesting feature of the LsHsp60 family is the variability in their intron phase. Based on the disruption of the last codon of an exon, introns have been divided into three types. The first is called phase 0, in which introns do not disrupt a codon. The second is called phase 1, in which introns disrupt a codon between the first and second bases. Finally, phase 2, where introns disrupt a codon between the second and third bases [16]. Unlike LsHsfs and the other LsHsps families, every LsHsp60 gene has at least one phase 0 and phase 2 intron, with the only exception of LsCpn60-8 which does not have any introns (Fig. 4C).

Tandem gene duplication of Lettuce Hsp70s

Hsp70s are molecular regulators of stress responses, as they maintain protein homeostasis by mediating folding or protein denaturation [17]. Hsp70 family is comprised of DnaK subfamily including cytoplasmic Hsp70 (Hsp70), truncated Hsp70 (Hsp70t), plastidic Hsp70 (cpHsp70), mitochondrial Hsp70 (mtHsp70), endoplasmic reticulum-localized immunoglobulin binding protein (BiP), and the Hsp110/SSE subfamily [18]. We identified nine Hsp110/SSE genes, one Hsp70t gene, three cpHsc genes, two mtHsc genes, and two BiP genes in lettuce (Fig. 5A). Strikingly, the lettuce genome contained 47 cytosolic Hsp70 genes (Fig. 5A-B), which greatly increased the number of the Hsp70 family genes (64 genes) compared with 18 genes found in Arabidopsis (Fig. 1A). From the 47 members of the cytosolic Hsp70, only three of them have orthologous in Arabidopsis, the remaining 44 members are novel lettuce Hsp60s (Fig. 5A-B). Another LsHsp70 subclass in which we identified new members was the LsHsp110/SSE (Fig. 5B). In Arabidopsis, four members of the Hsp110/SSE have been described. Our analysis yielded nine members, in which five are clustered together and do not have orthologues in Arabidopsis (Fig. 5B).

An interesting gene feature of the Hsp70 subfamily is the low number of exons and the well conserved motif organization. The LsHsp70t subclass single member has no introns and a few motifs, differently than all the other subclasses. Cytosolic Hsp70 genes have one intron and a well conserved motif distribution (Fig. 6A). However, some of the residues are not well conserved, except for motifs 5, 7, 8, 15, 16, 19, and 20 (Fig. 6B). Taken together, our results

show a large increase in gene number in the LsHsp70 family, which could be related to stress responsiveness to environmental signals.

Heat Shock Protein 90 and 100

Hsp90s and Hsp100s are the two heat shock protein subfamilies with the highest molecular weight (Fig. 1B). Unlike the other LsHsps families, both LSHsp90 and LSHsp100/ClpB families did not increase in gene number during the lettuce whole-genome triplication in comparison with Arabidopsis, rice, and poplar (Fig. 1A). Remarkably, 62% of the LsHsp100s were localized in chloroplasts, 26% in cytoplasm, and 12% were mitochondrial localized (Fig. 1B). LsHsp90s showed a more diverse localization in several subcellular compartments including ER, nucleus, cytoplasm and chloroplast as well as physiochemical features including MW, pI, and hydropathicity (Fig. 1B; Additional file 1: Table S1).

Plant Hsp90 family proteins have been classified into two main groups (group I and II), and are divided into four subclasses (group Ia, Ib, IIa, and IIb) [19]. Similarly, LsHsp90s subfamily contain seven members, organized in four subclasses. Group Ia includes LsHsp90-1a and LsHsp90-1b (Fig. 7A), both orthologous genes of a single member described in Arabidopsis. Similarly, group Ib contains only two members orthologous of AtHsp90-2/3/4 group (Fig. 7B). At the gene structure level, LsHsp90s can be divided in two main groups based on their exon and motif numbers (Fig. 7C). All the members of the group Ia and Ib have three exons and a highly similar motif structure. In contrast, members of the group IIa and IIb have a larger number of exons (19

and 12 exons, respectively) and a different motif pattern compared to the group Ia and Ib, but similar among them (Fig. 7C).

LsHsp100/ClpB family contains five subclasses (Fig. 7D), ClpB-Cyt (cytoplasmic localized), ClpB-CP (chloroplast localized), ClpB-MT (mitochondrial localized), ClpC, and ClpD. Phylogenetic analysis shows that lettuce and Arabidopsis shared a comparable number of group members with similar localization (Fig. 7E). Lettuce Hsp100 family genes also showed structural similarities depending on their subclasses. Cytoplasmic ClpB genes included only two exons; however, chloroplastic and mitochondrial ClpB, ClpC, and ClpD genes contained between eight to twelve exons (Fig. 7F).

Chromosomal distribution and duplication events of Hsf and Hsp genes in lettuce

In our initial analysis, we found an increased number of Hsfs and Hsps in lettuce compared to other species (Fig. 1A). To further investigate which particular Hsfs and Hsps subfamilies underwent duplication or genome loss, we first analyzed their chromosomal distribution. A total combined of 165 Hsfs and Hsps were randomly distributed in each of the nine lettuce chromosomes (Fig. 8). A syntenic analysis of lettuce against other plant species of the Asterid order suggested a whole-genome triplication event in lettuce since its divergence from the grape lineage [5]. Using syntenic regions previously categorized [5], we searched for Hsps and Hsfs located in those regions (Fig. 8). Remarkably, 43.7% (14 out of 32) of the LsHsfs were located in syntenic regions, suggesting their importance during the whole-genome triplication process. Syntenic region A was found in chr1, chr2, and chr6 and only contained Hsps subfamily 60 in particular of the subclass LsCpn60. Syntenic region B is present in chr1, chr4 and chr5 and

carries mostly members of the LsClp (LsHsp100). Syntenic region D carries LsHsfB1a in chr3 and LsHsfB1b in chr4. It was also present in chr2; however, no other LsHsf was located in that syntenic region (D). Syntenic region E was more heterogenous and it is present in chr2, chr8, and chr9. It carries LsHsfs and members of the LsHP70 and LsHsp90. Interestingly, syntenic region G was only located in chr3 carrying LsHsfA1A and LsHsfB2b, while chr6 carries LsBiP1 and LsBiP2 (LsHsp70). Syntenic region I contain LscpHsc70-1 and Lsmthsc70-1 in chr5 and their duplicated genes LscpHsc70-3 and Lsmthsc70-2 were found in chr9 respectively. Syntenic region H carries a diverse mix of LsHsfs and LsHsps and it is located in chr5, chr8 and chr9. Finally, syntenic regions C, F and H do not carry any Hsfs and Hsps.

To further elucidate whether LsHsps were localized as gene clusters within the lettuce genome, we searched for regions containing two or more Hsps genes within a 200 kb region (Additional file 4: Table S4). The large majority of gene clusters found in the lettuce genome represent genes within the LsHsp70s subfamily (Fig. 8), and are distributed in chr1, chr4, chr5, chr7, chr8, and chr9. Interestingly, chr1 and chr9 carry gene clusters with nine and ten LsHsps70s genes, respectively. This indicates the greater number of Hsp70 genes found in lettuce compared to Arabidopsis, rice, and poplar could be the result of clustered tandem duplication. Similarly, chr8 carries two gene clusters of LsHsp70s. A second group of abundant gene clusters found in the lettuce genome belong to the LssHsps family. Tandem repeat of LssHsps gene clusters were found in chr2, chr7, chr8, and chr9. Our results showed that LsHsfs and LsHsps70s were fundamental genes during the whole-genome triplication in lettuce.

Cis-element analysis of lettuce Hsf and Hsp genes

In order to understand how LsHsfs and LsHsps are regulated, we performed a Cis-regulatory element analysis of LsHsfs and LsHsps promoters using a 2 kb upstream region of each gene. We particularly focused on Cis-regulatory elements related to light, stress, and hormone responses as well as metabolism, cell cycle, and circadian rhythm (Fig. 9; Additional File 5: Table S5). A total number of 59 cis-regulatory elements were identified among LsHsfs and LsHsp genes. Interestingly, within the light responsive cis-elements, G-box, GT1 motif, TCT motif, and box 4 were highly abundant in all LsHsf and LsHsp gene families. ARE cis-element was the most abundant in the stress responsive category, having multiple ARE motif present across the promoter of both LsHsfs and LsHsps genes (Fig. 9; Additional File 5: Table S5). In the hormone stress category ABRE was ubiquitously present in the LsHsfs promoters and in most of the LsHsp promoters. However, ABRE motif was underrepresented in promoter regions of LsHsp90s (Fig. 9; Additional File 5: Table S5). Cis-elements related to metabolism, cell cycle and circadian rhythm were less abundant and indicate the low involvement of LsHsfs and LsHsps in these biological processes.

Transcriptional analysis of LsHsfs and LsHsps under different light conditions

Many studies have shown that Hsfs and Hsps genes are involved in plant responses to abiotic stresses in model plants [10] and other crop species [11, 14, 20, 21]. To investigate the role of LsHsfs and LsHsps under different light conditions, we grew lettuce plants *cv. Codex* in hydroponic conditions. After germination, lettuce plants were grown for 10 days under broad-spectrum LED lamps providing an average of $150 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light unit. Then, light conditions were adjusted to $220 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light for 22 days. Control plants were kept at

the same light conditions while UV treated plants were exposed to $220 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ supplemented with UV radiation. High light condition was achieved by providing $440 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. All end-of-production treatments were applied for 4 days and then mature lettuce plants were harvested for gene expression analysis (Fig. 10A). In general, lettuce plants under UV and high light conditions became reddish while lettuce leaves retained a green color under control conditions (Fig. 10B).

We selected representative members of each subfamily of LsHsfs and LsHsps based on our genome-wide analysis. In general, LsHsfs types A, B, and C were highly responsive to UV and high light stress. LsHsfA1 and LsHsfA7c, type A Hsfs, showed significant downregulation when lettuce plants were exposed to UV radiation and high light conditions (Fig. 10C). In contrast, LsHsfB1a was only downregulated in response to high light but did not respond to UV radiation. In the case of Hsfs type C, we analyzed the expression of LsHsfC1c and observed an upregulation in response only to UV radiation (Fig. 10C).

Almost all subclasses of LsHsps were upregulated in response to at least one of the treatments, UV radiation or high light. Interestingly, members of the LsHsp-CP and LsHsp-PX subclasses showed the highest upregulation in response to UV treatment (Fig. 10C). Transcriptional expression of LsHsp60s under UV radiation and high light showed a wide range of responses. Although LsCpn60-2, LsCpn60 α 4 LsCpn60-1 did not respond to neither of the treatments, LsCpn60-4a was upregulated and LsCpn60 α 3 downregulated for both treatments (UV radiation and high light) (Fig. 10C).

In relation of LsHsp70s, all the novel members identified were responsive to both treatments (UV and high light stress); however, high light was the most effective at downregulating their expression (Fig. 10C). Interestingly, members of the cpHsc and Hsp110/SSE subclasses did not show any transcriptional response. Remarkably, LsHsp90 and LsHsp100 did not respond to UV and high light treatment, suggesting that they might not be involved in response to light conditions.

Since we found tandem duplication of several LsHsps genes, we selected several of those genes to explore the possibility of cluster expression in response to light conditions. Surprisingly, we did not find any transcriptional response of duplicated genes based on our RT-qPCR data (Fig. 10C). Interestingly, LsHsp18.2-CII did not respond to light stress; however, LsHsp18.4b-CII located in the same duplicated cluster was significantly upregulated in response to high light. Another example was the case of LsCpn60 α 3 and LsCpn60 α 4 in which the former was significantly downregulated in response to both treatments, but the latter did not change transcriptionally (Fig. 10C). Because most of the new LsHsp70s members that we identified are located in clusters (Fig. 8), we tested three of these clusters containing LsHsp70s genes (Fig. 10C). We found that none of the genes tested show similar transcriptional pattern. It is worth noticing that functional characterization of those clusters might provide a better understanding of their regulatory role.

Discussion

Plant stress responses are frequently the result of simultaneous mechanisms that synergistically and/or coordinately operate to prevent, maintain, and re-establish cellular homeostasis [22–24]. One of the best characterized stress mechanisms is the heat shock proteins response. It has been demonstrated that Hsps play an important role in many cellular processes in response to stressful conditions, which may disclose whole plant responses to multiple environmental stresses. This particular stress response mechanism involves the activation of different Hsp families [17, 21] regulated by Hsfs to produce a rapid and constitutive response. Thus, the different classes of Hsps work together with Hsf to maintain cellular homeostasis [10, 17]. In this study, we performed a genome-wide analysis for Hsfs and Hsps in lettuce and identified an overall increase in most of these gene families.

Diversification of LsHsfs

The composition of the Hsfs family has so far been fully described in model plant species as well as in some cereal crops [10–12, 20, 25]. There are 21 Hsfs in *Arabidopsis* (*Arabidopsis thaliana*), 26 Hsfs in tomato (*Solanum lycopersicum*), 25 in both rice (*Oryza sativa*) and maize (*Zea mays*) and 52 in soybean (*Glycine max*) [13, 25–27]. However, several studies have shown that Hsfs is subject to evolutionary changes based on both number and composition of the Hsf family [27]. Our genome-wide analysis in lettuce identified 32 Hsfs genes; a higher number compared to *Arabidopsis* (Fig. 2A-B). This increase in gene number might have resulted in evolutionary advantage during the whole genome triplication [5]. Interestingly, unlike Hsps, almost all Hsfs are not located in tandem repeats in the lettuce genome, with the exception of LsHsf5b and

LsHsf5c which are positioned in tandem repeat in the chr7 (Fig. 8), suggesting that all LsHsfs are functional genes with a possible role in the stress response and homeostasis maintenance. The basic structure of Hsf proteins is formed by a DNA binding domain, oligomerization domain, and a C-terminal activation domain. Classification of plant Hsfs (A, B, and C) is mainly based on variances in these three domains, particularly the oligomerization domain. The most obvious differences are the insertion of additional amino acid residues in the oligomerization domain of type A and type C Hsfs and the absence of transcriptional activator motifs in the C-terminal domain of type B and C Hsfs [20, 25]. In Lettuce, all the Hsfs follow the same basic structure. For instance, the type C members lack C-terminal domains resulting in smaller genes (Fig. 2C). Type A Hsfs are known to function as transcriptional activators of stress genes. Genetic and functional analysis suggests that HsfA1a and HsfA1b are central regulators required in the early phase of the heat shock response [22, 24, 28]. In Arabidopsis, members of the Hsfs type A function as the main positive regulators in heat shock-responsive gene expression including members of the Hsp70 and Hsp90 families [29, 30]. We observed a strong downregulation of LsHsfA1a under UV and high light (Fig. 10), which might explain the lack of response of LsHsp90s and LsHsps100s to light stress conditions.

Duplications play major role in the diversification of the LsHsp70 family

In plants, gene duplication through whole-genome duplication (WGD), tandem or segmental events represent a major force driving gene family expansion [31, 32]. Similarly as described in many other plant species [10, 11], lettuce Hsfs and Hsps have experienced complex biological rearrangements, resulting in adaptation to specific conditions (Fig. 8) [5]. Our phylogenetic

analysis comparing LsHsp70s with Arabidopsis, rice, and poplar revealed six different groups, designated as cytosolic Hsp70, Hsp70t, BiP, MtHsc, cpHsc and Hsp110/SSE (Fig. 5B). We found that the LsHsp70 family was the one that had undergone the largest duplication within the Hsps. Out of 64 LsHsp70 gene members 55% (36 members) are localized in duplicated blocks in the lettuce genome. Our findings suggest that prior to the whole genome triplication, LsHSP70 suffered dynamic rearrangements that allowed the emergence of novel members of the LsHsp70 family (Fig. 5). Thus, the ancient genetic rearrangement in the LsHsp70 family might have resulted from the whole-genome triplication as an essential genetic group of this plant species. Interestingly, 39% of the LsHsp70s have a single exon, all of them from the highly abundant cytosolic Hsp70 subclass (Fig. 6), which is in line with previous studies which suggest that the evolution of several plant gene families is associated with the diversification of exon and intron within those gene family members [33].

Light quality effects on LsHsfs and LsHsps transcriptional levels

During a plant life's cycle, a large number of essential processes are regulated by light. These processes include photosynthesis, plant growth and development, anthocyanin biosynthesis, and gene expression, among others [2, 3, 34, 35]. In Arabidopsis, three A-type Hsfs, HsfA1D, HsfA2, and HsfA3 were found to regulate early responses during excess light energy [36]. In lettuce, under UV and high light, we found strong downregulation of LsHsfA1a and LsHsfA7c (Fig. 10C). In addition, LsHsfB1a was downregulated in response to high light and LsHsfC1c upregulated in response to UV. Interestingly, under different light conditions, most studied members of the Lettuce Hsp90 and Hsp100 families were not regulated, while members of the

LssHsp and LsHps60 families were significantly up-regulated (Fig. 10C). While further studies need to be conducted to elucidate the functions of these genes under UV and high light conditions, our data suggests that the hydroponic conditions we used in this study are not able to trigger LsHsp90s and LsHsp100s molecular responses.

The specific response to UV and high light of many members of the LsHsp70 family might be associated with the increased member duplication of the cytosolic Hsp70 in lettuce. Under excessive light energy, plants first try to dissipate excess light stored as electron energy in the chloroplasts to avoid heat damage [37]. Under UV conditions, we observed a high transcriptional expression of Hsfs and Hsps. For instance, a strong downregulation of LsHsp70-28 and LsHsp70t-1 specially to high light conditions but a significant upregulation of these under UV (Fig. 10C). Our study provides initial assessment of Hsfs and Hsps under different light conditions, However, the precise regulatory mechanisms under hydroponic conditions under control and stress conditions require further investigation.

Conclusions

Our detailed genome-wide analysis on the heat shock factors and heat shock proteins in lettuce identified 32 and 165 genes, respectively. Phylogenic analysis Hsfs and Hsps genes highlight a close relationship with their orthologous genes in Arabidopsis. Interestingly, a large number of novel members were also found in Hsfs, sHsps and Hsp70 gene families probably as result of the genome wide triplication. Several Hsfs, sHsps, Hsp60s, and Hsp70s genes are highly responsive to UV and high light conditions and provide candidates for breeding programs

aiming to produce lettuce varieties able to grow healthy under hydroponic systems that use artificial light.

Methods

Plant material and growth conditions

Lettuce seeds of red-leaf cultivar cv. *Codex rz* were pre-germinated for 48 hours until radicle emergence was observed. Germinated seeds were transplanted into rockwool plugs [2]. Seedlings were propagated for 10 days inside a walk-in growth chamber using an average photosynthetic photon flux of $150 \pm 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (24-h photoperiod) and a daily light integral (DLI) of $12.96 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ provided by broad-spectrum LED lamps (Philips GP150 Red/Deep Red/Blue Low Blue High Output). Temperature and relative humidity (RH) were set at 24°C and 60% to 80%, respectively; CO₂ concentration was maintained at ambient levels. Seedlings of similar size were transplanted into individual deep-water culture closed hydroponic systems using plastic cups of 5 cm of diameter. Plants were grown using a commercial water-soluble fertilizer (OASIS® Grower Solutions Hydroponic Fertilizer 16-4-17) dissolved in tap water at a concentration of 150 mg·L⁻¹ N (EC and pH = approx. 1.2 dS·m⁻¹ and 6.0, respectively). Each 7.6 L hydroponic system had a clear plastic tube attached to an air pump to provide continuous aeration.

Light treatments

Lettuce plants were grown inside a growth chamber equipped with two opposite multi-level shelves. Each shelf was considered a block with four levels used as treatment compartments.

All compartments held four hydroponic systems. Plants were grown under broad-spectrum LED lamps providing an average DLI of $15.84 \text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ ($220 \pm 5 \text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 20-h photoperiod from 02:00 to 22:00 h). The average ambient day (from 02:00 to 22:00 h) and night (from 22:00 to 02:00 h) air temperature of the chamber was set at 22°C and 21°C, respectively; CO₂ concentration, and RH was set at 405 ppm and 60% to 80%, respectively. After 22 days in the chamber, plants were subjected to one of three treatments during 4 days: end-of-production (EOP) UV-A ($11 \text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) + white light ($220 \text{ }\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$), EOP high light ($440 \pm 5 \text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and a control ($220 \pm 5 \text{ }\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) with no EOP treatment (Fig. 10A).

Sequence retrieval and phylogenetic reconstruction

Heat Shock Factors (Hsfs) and Heat Shock Proteins (Hsps) sequences of Arabidopsis, rice and poplar [10–12] were retrieved from public databases. When a gene had splice variants, primary transcripts were selected as representative for the gene. To identify putative lettuce *Hsfs* and *Hsps*, retrieved sequences were used as queries using an automated BLASTP search against the lettuce genome database in Phytozome [38]. An E-value threshold of 1E^{-20} was used. When multiple transcripts were predicted for a locus from BLASTP, a transcript with the lowest E-value was chosen as a representative for the locus.

Phylogenetic trees were generated by the Maximum Likelihood method and Le_Gascuel_2008 model [39] with 500 Bootstrap replications using MEGA X software [40] as previously described [41]. A discrete Gamma distribution was used to model evolutionary rate differences among sites. Gene names of lettuce Hsfs and Hsps were assigned based on their phylogenetic relationship with Arabidopsis proteins. Lettuce sHsps were classified according to their

molecular weights obtained using the ProtParam tool [42]. Phylogenetic analysis data was deposited in the repository of phylogenetic information, TreeBASE and it is available in the following link: <http://purl.org/phylo/treebase/phyloids/study/TB2:S27434?x-access-code=f2368699e8e38ce6d23cad64f44dc452&format=html>

Physical and chemical properties of Heat shock factors and heat shock proteins

The physical and chemical properties including molecular weight, isoelectric point, instability index, aliphatic index, and hydropathicity of all lettuce Hsfs and Hsps were estimated using ProtParam tool from ExPASy server [42]. Protein subcellular localization of lettuce Hsfs and Hsps were predicted using WoLF PSORT [43]. Multiple sequence alignment for Arabidopsis and lettuce Hsfs and Hsps were performed by MUSCLE [44].

Chromosomal mapping and characterization of tandem duplication events of Hsf and Hsp genes in the lettuce genome

The chromosomal locations of Lettuce Hsfs and Hsps genes were retrieved from Phytozome, *Lactuca sativa* V8 genome database [38]. Homologous chromosome segments resulting from whole-genome triplication events were identified as described previously [5]. Segmental gene duplication events were represented according to the phylogenetic relationship among adjacent genes and sequence similarity ($\geq 70\%$). Tandem duplication was evaluated based on the following criteria: genes within a 200-kbp region in a chromosome with the nucleotide sequence similarity $\geq 70\%$. Sequence similarity was calculated using the EMBOSS Water with Smith-Waterman local alignment algorithm [45].

480

481 **Non-synonymous (dN) to synonymous substitution (dS) ratio (dN/dS) analysis**

482 The Ratio between non-synonymous mutations (dN) to synonymous mutation (dS) of each
483 lettuce Hsf or Hsp gene and their corresponding Arabidopsis ortholog was calculated using
484 EMBOSS Water pairwise alignment [45]. The dN/dS ratio was estimated by utilizing the
485 PAL2NAL tool with the input of the pairwise DNA sequence alignment [46].

486

487 **Gene structure, conserved motif and Cis-regulatory element analysis of LsHsf and LsHsp genes**

488 Exon-intron structures of lettuce Hsfs and Hsps were depicted using the Gene Structure Display
489 Server – GSDS v2.0 [47]. Genomic sequence and coding sequences of each Hsf or Hsp gene
490 were downloaded from Phytozome database and aligned to predict the exon-intron structure.

491 Conserved motifs analysis of Hsfs and each Hsp family were determined using Multiple Em for
492 Motif Elicitation (MEME) Suite online program with the following parameters: maximum motif
493 numbers = 20; site distributions = any number of repetitions; motif width = 6 to 50 [48].

494 Cis-regulatory elements of Lettuce Hsf and Hsp genes were identified using 2,000 bp upstream
495 regions of each lettuce Hsf and Hsp genes using the PlantCARE database [49].

496

497 **RNA isolation and RT-qPCR**

498 Total RNA from leaves of twenty-two days old mature plants was isolated using Trizol (Ambion)
499 following the manufacturer's instructions. cDNA synthesis was performed using reverse
500 transcription system (Invitrogen SuperScript II) and oligo(dT) primers. Real-time PCR reactions
501 were performed using SYBR Green FastMix (Quantabio) as previously described [50]. Lettuce

Tubulin (LsTUB) was used as housekeeping gene for internal normalization control. qPCRs were performed in the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems) in a 96-well reaction plate. Primers used are described in Additional File 6: Table S6. Cycling parameters consisted of 5 min at 95°C, and 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s [51]. RT-qPCR reactions were performed in triplicate for each RNA sample on three biological replicates of each light condition. Specificity of the amplifications was verified by a melting curve analysis. Relative amounts of mRNA were calculated from threshold points (Ct values) located in the log-linear range of real-time PCR amplification plots using the $2^{-\Delta\Delta C_t}$ method [52].

Statistical analysis

R software/environment was used for the statistical analyses of the RT-qPCR data. Three independent experiments in which each experiment had four biological replicates were used. t-test was used to compare gene expression between the experimental groups (UV and high light) and the control group (normal light). Differences in means were considered significant at p-value < 0.05.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

523 All data generated or analyzed during this study are included in this published article and its
524 supplementary information files.

525 **Competing interests**

526 The authors declare that they have no competing interests

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531 **Authors' contributions**

532 T.K. and S.S performed the bioinformatics analysis. T.K. and J.J performed the experiments. C.G.
533 provided plant material. T.L provided equipment and reagents. K.B. conceived the experiments.
534 T.K and K.B. analyzed the data. T.K. prepared the figures. KB wrote the manuscript with input
535 from T.K. All authors read and approved the manuscript.

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538 **Authors' information (optional)**

539 T.K; S.S; J.J; C.G and K.B from University of Florida, Environmental Horticulture Department,
540 Gainesville, Florida. T.L from University of Florida, Horticultural Science Department,
541 Gainesville, Florida.

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Figure Legends

Fig. 1. General gene structure features of lettuce Hsfs and Hsps. (A) Numbers of Hsf and Hsp genes in lettuce, Arabidopsis, poplar and rice. (B) Molecular weight analysis of LsHsfs and LsHsps. (C) Exon number distribution of the lettuce Hsfs and Hsps. (D) Evaluation of positive selection of LsHsfs and LsHsps families based on the dN/dS ratio analysis.

Fig. 2. Phylogenetic analysis, exon-intron structure, and motif distribution of lettuce *heat shock transcription factor (LsHsf)* gene family. (A) List of lettuce Hsf genes. (B) Phylogenetic relationship of Hsf proteins of lettuce and Arabidopsis. *LsHsf* and *AtHsf* members in each subfamily (Type A, Type B, and Type C) are represented in different colors. (C) Gene structure and motif composition analysis of *LsHsf* genes with blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Fig. 3. Identification, phylogenetic and gene structure analysis of lettuce *small heat shock protein (LssHsp)* gene family. (A) List of sHsp genes in lettuce. Classification of *LssHsp* genes were assigned according to their molecular weight and subcellular localization (Additional Table S1). (B) Phylogenetic relationship of LsHsp proteins and *A. thaliana*. LssHsp proteins are marked in bold. LssHsp and AtsHsp members in each subfamily (C-I, C-II, C-III, C-IV, C-V, C-VI, ER, MT, CP, and PX) are represented in different colors. (C) Gene structure and motif composition analysis of *LssHsp* genes. Blue boxes, green boxes, and black lines indicate untranslated region (UTR),

coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Fig. 4. Classification, evolutionary relationship, gene structure and motif distribution of lettuce *heat shock protein 60* (*LsHsp60*) gene family. (A) List of lettuce Hsp60 genes. The nomenclature of *LsHsp60* genes were assigned according to the closest *AtHsp60* genes. (B) Phylogenetic relationship of Hsp60 proteins of *L. sativa* and *A. thaliana*. *LsHsp60* proteins are marked in bold. *LsHsp60* and *AtHsp60* members in each subfamily (Cpn60, Cpn60 α , Cpn60 β , and Hsp60) are represented in different colors. (C) Gene structure and motif composition analysis of *LsHsp60* genes. Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Fig. 5. Phylogenetic analysis, exon-intron structure, and motif distribution of lettuce *heat shock protein 70* (*LsHsp70*) gene family. (A) List of Hsp70 genes in lettuce. Homologous transcripts of lettuce Hsp70s were identified by BLASTP search using *A. thaliana* Hsp70 protein sequences. The nomenclature of *LsHsp70* genes were assigned according to the name of *AtHsp70* gene of the highest similarity. (B) Phylogenetic relationship of Hsp70 proteins of *L. sativa* and *A. thaliana*. *LsHsp70* proteins are marked in bold. *LsHsp70* and *AtHsp70* members in each subfamily (Hsp70, Hsp70t, mtHsc, cpHsc, BiP, and Hsp110/SSE) are represented in different colors.

Fig. 6. Gene structure and motif composition analysis of *LsHsp70* genes. (A) Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). (B) Conserved motif analysis of *LsHsp70* proteins; 20 identified motifs are shown. Detailed information of all motifs is shown in Additional file 3: Table S3.

Fig. 7. Characterization of lettuce *heat shock protein 90 (LsHsp90)* and *heat shock protein 100 (LsHsp100)* gene families. (A) Gene members of the *Hsp90* family in lettuce (B). Phylogenetic tree of *Hsp90* proteins of *L. sativa* and *A. thaliana*. Lettuce *Hsp* proteins are marked in bold. (C) Gene structure and motif composition analysis of *LsHsp90*. (D) Gene members of the *LsHsp100* family. (E) Phylogenetic analysis of *Hsp100* proteins of *L. sativa* and *A. thaliana*. (F). Gene structure and motif composition analysis of *LsHsp100*. Nomenclature of *LsHsp90* and *LsHsp100* genes were assigned according to the name of *A. thaliana* homologues. Lettuce *Hsp90* and *Hsp100* family members are represented in different colors according to their subfamily (Group Ia, Ib, IIa, and IIb in *Hsp90* ClpB-Cyt, ClpB-MT, ClpB-CP, ClpC, and ClpD in *Hsp100*). (C, F) Exon-intron organization. Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Fig. 8. Genome-wide distribution of lettuce triplicated regions highlighting chromosomal location of Hsfs and Hsps genes. Lettuce syntenic regions are shown with colored blocks in each chromosome (LG1 to LG9). Physical location of each *LsHsf* or *LsHsp* gene is shown and gene names are colored according to their gene family. *LsHsfC1a* (*Lsat_1_v5_gn_0_11820*) was

not included because it has not been mapped on any of the nine chromosomes in lettuce.

Boxes represent tandemly duplicated genes. Paralogous gene pairs originated from segmental duplications are highlighted with circles and connected by lines.

Fig. 9. Cis-regulatory element analysis of lettuce Hsf and Hsp genes. Putative cis-regulatory elements were analyzed in the 2 kb upstream regions of *LsHsf* and *LsHsp* genes. Six groups of cis-regulatory elements (light response, stress response, hormone response, metabolism, cell cycle, and circadian rhythm) are displayed. Colors from white to red indicate the occurrence of each cis-regulatory element from 0 to 10+ (10 or more).

Fig. 10. Gene expression analysis of lettuce Hsf and Hsp genes under different light conditions. (A) Schematic representation of the experimental design used at different light treatment on lettuce plants. *L. sativa* cultivar Codex plants were grown under light $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 10 days then $220 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was treated for 22 days. Control light ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), UV light ($11 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ supplemented with $220 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of white light), and high light ($440 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were imposed to lettuce plants for 4 days. (B) Physical appearance of *L. sativa* cultivar “Codex” 4 days after treatment of control light (CT), UV, and high light (HL). (C) The relative expression of lettuce Hsf and Hsp genes. mRNA levels were determined by qRT-PCR analysis and normalized to that of *LsTUB*. $\log_2\text{FC}$ (fold change) values of relative expression of treatment (UV and HL) to the control (CT) are presented. Asterisks indicate significant differences between treatment and control determined by Student’s t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Genes marked with the same letter (a-e) belong to the same tandem duplicated gene cluster.

632 Additional Table S1. General properties.

633 Additional Table S2. KaKs ratio

634 Additional Table S3. MEME motif

635 Additional Table S4. Tandem and segmental duplication

636 Additional Table S5. Cis-element analysis

637 Additional Table S6. Primers used for RT-qPCR analysis

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Figures

Figure 1

General gene structure features of lettuce Hsfs and Hsps. (A) Numbers of Hsf and Hsp genes in lettuce, Arabidopsis, poplar and rice. (B) Molecular weight analysis of LsHsfs and LsHsps. (C) Exon number distribution of the lettuce Hsfs and Hsps. (D) Evaluation of positive selection of LsHsfs and LsHsps families based on the dN/dS ratio analysis.

Figure 2

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intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Identification, phylogenetic and gene structure analysis of lettuce small heat shock protein (LssHsp) gene family. (A) List of sHsp genes in lettuce. Classification of LssHsp genes were assigned according to their molecular weight and subcellular localization (Additional Table S1). (B) Phylogenetic relationship of

LsHsp proteins and A. thaliana. LssHsp proteins are marked in bold. LssHsp and AtsHsp members in each subfamily (C-I, C-II, C-III, C-IV, C-V, C-VI, ER, MT, CP, and PX) are represented in different colors. (C) Gene structure and motif composition analysis of LssHsp genes. Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Figure 4

Classification, evolutionary relationship, gene structure and motif distribution of lettuce heat shock protein 60 (LsHsp60) gene family. (A) List of lettuce Hsp60 genes. The nomenclature of LsHsp60 genes were assigned according to the closest AtHsp60 genes. (B) Phylogenetic relationship of Hsp60 proteins of *L. sativa* and *A. thaliana*. LsHsp60 proteins are marked in bold. LsHsp60 and AtHsp60 members in each subfamily (Cpn60, Cpn60α, Cpn60β, and Hsp60) are represented in different colors. (C) Gene structure and motif composition analysis of LsHsp60 genes. Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Figure 5

Phylogenetic analysis, exon-intron structure, and motif distribution of lettuce heat shock protein 70 (LsHsp70) gene family. (A) List of Hsp70 genes in lettuce. Homologous transcripts of lettuce Hsp70s were identified by BLASTP search using *A. thaliana* Hsp70 protein sequences. The nomenclature of

LsHsp70 genes were assigned according to the name of AtHsp70 gene of the highest similarity. (B) Phylogenetic relationship of Hsp70 proteins of *L. sativa* and *A. thaliana*. LsHsp70 proteins are marked in bold. LsHsp70 and AtHsp70 members in each subfamily (Hsp70, Hsp70t, mtHsc, cpHsc, BiP, and Hsp110/SSE) are represented in different colors.

Figure 6

Gene structure and motif composition analysis of LsHsp70 genes. (A) Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). (B) Conserved motif analysis of LsHsp70 proteins; 20 identified motifs are shown. Detailed information of all motifs is shown in Additional file 3: Table S3.

Figure 7

Characterization of lettuce heat shock protein 90 (LsHsp90) and heat shock protein 100 (LsHsp100) gene families. (A) Gene members of the Hsp90 family in lettuce (B). Phylogenetic tree of Hsp90 proteins of *L. sativa* and *A. thaliana*. Lettuce Hsp proteins are marked in bold. (C) Gene structure and motif composition analysis of LsHsp90. (D) Gene members of the LsHsp100 family. (E) Phylogenetic analysis of Hsp100 proteins of *L. sativa* and *A. thaliana*. (F). Gene structure and motif composition analysis of LsHsp100. Nomenclature of LsHsp90 and LsHsp100 genes were assigned according to the name of *A. thaliana* homologues. Lettuce Hsp90 and Hsp100 family members are represented in different colors according to their subfamily (Group Ia, Ib, IIa, and IIb in Hsp90ClpB-Cyt, ClpB-MT, ClpB-CP, ClpC, and ClpD in Hsp100. (C, F) Exon-intron organization. Blue boxes, green boxes, and black lines indicate untranslated region (UTR), coding sequence (CDS), and intron, respectively. The numbers on each intron represent the intron phase (0, 1, or 2). Detailed information of all motifs is shown in Additional file 3: Table S3.

Figure 8

Genome-wide distribution of lettuce triplicated regions highlighting chromosomal location of Hsfs and Hsps genes. Lettuce syntenic regions are shown with colored blocks in each chromosome (LG1 to LG9). Physical location of each LsHsf or LsHsp gene is shown and gene names are colored according to their gene family. LsHsfC1a (Lsat_1_v5_gn_0_11820) was not included because it has not been mapped on

any of the nine chromosomes in lettuce. Boxes represent tandemly duplicated genes. Paralogous gene pairs originated from segmental duplications are highlighted with circles and connected by lines.

Figure 9

Cis-regulatory element analysis of lettuce Hsf and Hsp genes. Putative cis-regulatory elements were analyzed in the 2 kb upstream regions of LsHsf and LsHsp genes. Six groups of cis-regulatory elements (light response, stress response, hormone response, metabolism, cell cycle, and circadian rhythm) are

displayed. Colors from white to red indicate the occurrence of each cis-regulatory element from 0 to 10+ (10 or more).

Figure 10

Gene expression analysis of lettuce Hsf and Hsp genes under different light conditions. (A) Schematic representation of the experimental design used at different light treatment on lettuce plants. *L. sativa* cultivar Codex plants were grown under light 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 10 days then 220 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was

treated for 22 days. Control light ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), UV light ($11\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ supplemented with $220\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of white light), and high light ($440\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were imposed to lettuce plants for 4 days. (B) Physical appearance of *L. sativa* cultivar “Codex” 4 days after treatment of control light (CT), UV, and high light (HL). (C) The relative expression of lettuce Hsf and Hsp genes. mRNA levels were determined by qRT-PCR analysis and normalized to that of LsTUB. log2FC (fold change) values of relative expression of treatment (UV and HL) to the control (CT) are presented. Asterisks indicate significant differences between treatment and control determined by Student’s t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Genes marked with the same letter (a-e) belong to the same tandem duplicated gene cluster.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.ListofHsfandHspgenesandtheircharacteristics.xlsx](#)
- [TableS2.dNdSratiobetweenLettucegenesandArabidopsishomologs.xlsx](#)
- [TableS3.MEMEMotifanalysis.xlsx](#)
- [TableS4.TandemSegmentalduplication.xlsx](#)
- [TableS5.CiselementanalysisofLsHsfandLsHsp.xlsx](#)
- [TableS6.PrimersusedintheqRTPCRanalysis.xlsx](#)