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Ultra-fast alterations in mRNA levels uncover multiple players in light stress acclimation in plants

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Accession numbers: Arabidopsis Genome Initiative locus identifiers for genes mentioned in this article are as follows: Zat12 (At5g59820), WRKY18 (At4g31800), WRKY40 (At1g80840), APX1 (At1g07890), RbohD (At5g47910), Cat2 (At4g35090), GNAT family protein (At2g39030), KMD1 (At1g80440), EF1-a (At5g60390), proteins of unknown function (At5g10695 and At3g10020), a Golgi-associated protein (At5g51430), a RAV transcription factor (At1g25560) and a glycosyl hydrolase (At3g13750). RNA-Seq data for this work are deposited under the GEO accession number GSE60865.

SUMMARY

The acclimation of plants to changes in light intensity requires rapid responses at several different levels. These include biochemical and biophysical responses as well as alterations in the steady-state level of different transcripts and proteins. Recent studies utilizing promoter::reporter constructs suggested that transcriptional responses to changes in light intensity could occur within seconds, rates for which changes in mRNA expression are not routinely measured or functionally studied. To identify and characterize rapid changes in the steady-state level of different transcripts in response to light stress we performed RNA sequencing analysis of *Arabidopsis thaliana* plants subjected to light stress. Here we report that mRNA accumulation of 731 transcripts occurs as early as 20–60 sec following light stress application, and that at least five of these early response transcripts play an important biological role in the acclimation of plants to light stress. More than 20% of transcripts accumulating in plants within 20–60 sec of initiation of light stress are H_2O_2- and ABA-response transcripts, and the accumulation of several of these transcripts is inhibited by transcriptional inhibitors. In accordance with the association of rapid response transcripts with H_2O_2 and ABA signaling, a mutant impaired in ABA sensing (*abi-1*) was found to be more tolerant to light stress, and the response of several of the rapid response transcripts was altered in mutants impaired in reactive oxygen metabolism. Our findings reveal that transcriptome reprogramming in plants could occur within seconds of initiation of abiotic stress and that this response could invoke known as well as unknown proteins and pathways.

Keywords: ***Arabidopsis thaliana*, transcription, RNA-Seq, light stress, ultra-fast.**

INTRODUCTION

Playing a principal role in sustaining life on Earth, plants convert solar radiation into bio-available energy. Unable to avoid abiotic stress by means of relocation, plants have evolved sophisticated acclimation mechanisms to cope with changes in their environment. These include sensing, signal transduction and stress protection proteins and pathways (Nakashima and Yamaguchi-Shinozaki, 2006; Bailey-Serres and Voesenek, 2008; Cavanagh *et al.*, 2008; Munns and Tester, 2008; Chinnusamy and Zhu, 2009; Mittler and Blumwald, 2010). Although changes in environmental conditions could occur within seconds in nature, studies attempting to dissect the responses of plants to abiotic stress have traditionally focused on events occurring 10–30 min or hours after application of abiotic stress (Mittler *et al.*, 2012). Recent studies have nonetheless indicated that the response of plants to abiotic stress could occur much faster than previously measured, and that changes in environmental conditions such as temperature

or light intensity could cause immediate alterations in the level or structure of different proteins, metabolites and RNA molecules, as well as changes in the redox status of different molecules (Mittler, 2002; Miller *et al.*, 2009; Mittler *et al.*, 2012; Suzuki *et al.*, 2013a; Gilroy *et al.*, 2014; Dietz, 2015). Even a gradual change in environmental conditions could trigger a rapid response once a particular physiological or biochemical sensing threshold is passed (Mittler *et al.*, 2012). Alterations in metabolites and RNA species could result from stress-induced differential enzymatic coefficiencies, RNA transcription and processing, or metabolite and RNA stability. These could in turn reprogram the cell metabolome and transcriptome and trigger specific sensors for abiotic stress response that would in turn activate multiple signal transduction pathways and result in the mounting of a full-scale acclimation response (Mittler *et al.*, 2012). Although much is known about the different signaling and downstream pathways that mediate the acclimation of plants to stress, virtually nothing is known about the rapid changes in the metabolome and transcriptome of plants that occur within seconds to minutes of initiation of abiotic stress (Mittler *et al.*, 2012; Suzuki *et al.*, 2013a,b; Gilroy *et al.*, 2014; Miller *et al.*, 2009).

We recently reported on the detection of ultra-fast changes in the metabolome of plants subjected to high-light stress, with changes in many metabolites occurring as early as 15 sec after the application of light stress (Suzuki *et al.*, 2013a). Using a Zat12::luciferase reporter system, we also reported on the existence in plants of a rapid local and systemic signal termed the reactive oxygen species (ROS) wave that is activated by different abiotic stresses and propagates at rates of up to 8.4 cm min^{-1} (Miller *et al.*, 2009; Mittler *et al.*, 2011). Those reports, as well as a recent report on the existence of a rapid local and systemic 'calcium wave' (Choi *et al.*, 2014), and the possible integration of the two (Gilroy *et al.*, 2014), suggest that transcription, RNA stability and/or RNA processing responses in plants could occur at a much faster rate than is typically studied. Moreover, if such rapid responses occur, and have an important biological function, then the lack of data for early time points in many of the abiotic stress-response transcriptome studies deposited in different gene and data banks (e.g. Hruz *et al.*, 2008; https://genevestigator.com/gv/) could hamper our attempts to develop crops with enhanced tolerance to abiotic stresses because many of the important early response genes will be missed. In support of the possibility that the steady-state transcript level of many genes could be enhanced within seconds of initiation of abiotic stress are measurements of transcription rates in eukaryotic cells nearing or exceeding 50 kb min^{-1} (Maiuri *et al.*, 2011), and the discovery that many genes in eukaryotic cells contain stalled RNA polymerases at their promoters and could mount a rapid transcriptional response following changes in environmental conditions (Nechaev and Adelman, 2008; Levine, 2011; Kwak and Lis, 2013).

Here we uncover the ultra-fast transcriptome response of plants triggered by light stress. This response includes the ordered and clustered mRNA accumulation of 731 transcripts that occurs as early as 20–60 sec after application of light stress. We further determined that five of the transcripts involved in this ultra-fast response play an important biological role in the acclimation of plants to light stress, that many of the ultra-fast light stress-response transcripts are H_2O_2- or ABA-response transcripts, and that the accumulation of several of the ultra-fast response transcripts is inhibited by transcriptional inhibitors. We also report that a mutant impaired in ABA sensing (*abi-1*) is more tolerant to light stress, and that the response of several of the rapid response transcripts was altered in mutants impaired in ROS metabolism/signaling. Our findings reveal that transcriptome reprogramming in plants occurs at a much faster rate than is typically studied and that this response could involve known as well as unknown transcripts and pathways. Because several of the genes identified as ultra-fast light response genes appear to be important for acclimation to light stress, our studies highlight the need to study the ultra-fast transcriptional response of plants to other abiotic or biotic stresses that may include important, but as-yet unidentified, genes for acclimation to abiotic stress and/or plant resistance to pathogens and pests.

RESULTS

RNA sequencing (RNA-Seq) analysis of the ultra-fast response of Arabidopsis to light stress

Arabidopsis thaliana plants subjected to light stress for 0, 20, 30, 60 and 90 sec were used for transcriptome (RNA-Seq; 0, 20, 60 sec) and quantitative (q)PCR analyses and H_2O_2 measurements (0, 20, 30, 60 or 90 sec) in three biological replicates (Figure 1 and Figure S1a in Supporting Information). Each biological replicate contained three technical replicates of 15–20 plants each, grown in pots for 18–21 days, exposed to a light intensity of 1000 μmol m^{-2} sec^{-1} at 21°C, and immediately dipped in liquid nitrogen. The steady-state level of 731 transcripts was found to be significantly enhanced in response to light stress within 20–60 sec. These were divided into three clusters based on their expression pattern (Figure 1a, Tables S1–S3), indicating a complex response pattern that involved differential timing. Out of the 731 transcripts shown in Figure 1, 49 were found not to be annotated on the ATH1 Affymetrix chips (Table S4). Interestingly, only 34 transcripts out of the 731 shown in Figure 1(a) increased in expression from a very low basal level [below 2.0 fragments per kilobase of transcript per million fragments mapped (FPKM); Table S5]. The steady-state level of 419 and 668 transcripts

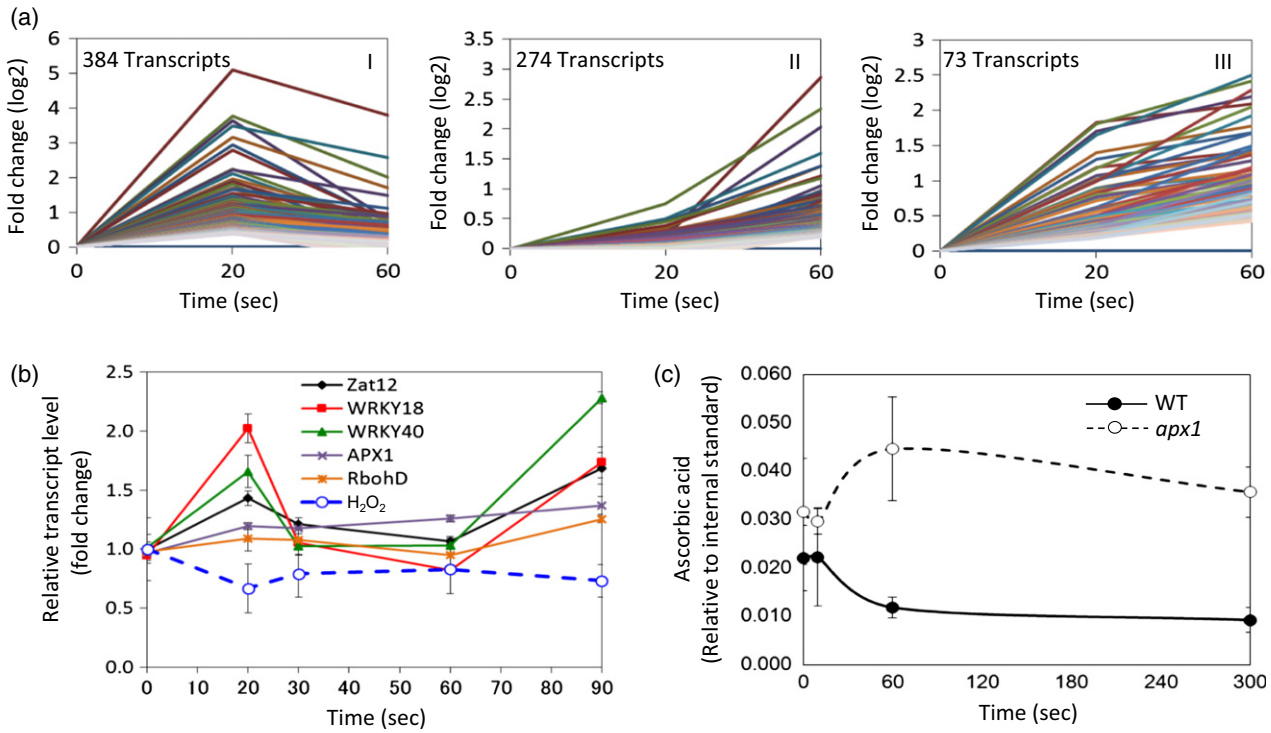


Figure 1. Ultra-fast alterations in transcript steady-state level in response to light stress in *Arabidopsis thaliana* detected by RNA sequencing.
(a) Three different clusters of transcript alterations distinguished by their pattern of response to light stress.
(b) Accumulation of H_2O_2 and selected transcripts determined by quantitative PCR during the early stages of light stress acclimation.
(c) Rapid changes in the level of ascorbic acid in response to light stress in wild-type (WT) plants and knockout mutants lacking the ascorbate-dependent H_2O_2-scavenging enzyme ascorbate peroxidase 1 (*apx1*).

significantly declined at 20 and 60 sec of light stress, respectively (Tables S6 and S7). As shown in Figure 1(b), qPCR analysis confirmed the expression of selected transcripts from Cluster I. It should be noted that few transcripts included in Cluster I in some experiments could be found in Clusters II or III in other experiments. These differences, detected by qPCR, could result from small variations in the physiological pre-conditioning of plants used for the different experiments or from the stochastic nature of the transcriptional response in different cells belonging to the same tissue (Stegle *et al.*, 2015). Interestingly, enhanced cellular levels of H_2O_2 were not detected during early stages of light stress (Figure 1b). This finding could be linked to the high content of antioxidants in plants, generating a buffer against rapid changes in ROS (Mittler, 2002; Halliwell, 2006). In accordance with this hypothesis, the level of ascorbic acid rapidly decreased in response to light stress in Arabidopsis in a process that was dependent on the presence of ascorbate peroxidase 1 (APX1; Davletova *et al.*, 2005a), an ascorbate-dependent H_2O_2-scavenging enzyme (Figure 1c). This finding demonstrated that APX1 could be directly involved in scavenging of H_2O_2 produced during the initial response to light stress, and that during this process the stored levels of ascorbic acid are utilized as part of the Asada–Foyer–Halliwell pathway (Figure 1c; Halliwell, 2006).

The majority of transcripts belonging to all ultra-fast response clusters were annotated as stress, abiotic or biotic response transcripts based on their Gene Ontology (GO) annotation (Figure 2a), and genes encoding the bulk of these transcripts were scattered on all Arabidopsis chromosomes, with only a few clustering at certain locations (Figure S1b). A breakdown of the overlap between responses to different biotic/abiotic stresses and the different transcripts found in each of the ultra-fast response cluster indicated that many of the transcripts with an enhanced steady-state level in response to light stress are also responsive to abiotic stresses such as drought, cold, heat and salinity, demonstrating their possible involvement in tolerance of abiotic stress in plants (Figure 2b; ATH1 chip data were obtained from the supplementary material of Huang *et al.*, 2008; Matsui *et al.*, 2008; Larkindale and Vierling, 2008; Kleine *et al.*, 2007; Ding *et al.*, 2014; Tosti *et al.*, 2006; Consales *et al.*, 2012; Truman *et al.*, 2006). A comparison, shown in a Venn diagram in Figure 2(c), between the transcriptomes of the ultra-fast response to light stress (this work), the response of Arabidopsis to H_2O_2 (Davletova *et al.*, 2005b; data obtained

from the supplementary material therein) and the response of Arabidopsis to a 3-h light treatment (Kleine *et al.*, 2007; data obtained from the supplementary material therein), revealed that 502 transcripts found to be induced between 20 and 60 sec after light stress application were not found to be induced by the longer light stress or H_2O_2 treatments. Out of these 502 transcripts, 245 transcripts (Table S8) were also not found to be included in the response of plants to the stresses shown in Figure 2(b). Of course, 49 of these were not represented in the ATH1 chips (Table S4). These transcripts could therefore represent a unique group of transcripts that are relatively more specific to the ultra-fast response (Table S8).

Functional characterization of ultra-fast response transcripts in Arabidopsis

To determine whether some of the ultra-fast response transcripts play a biological role in the acclimation of plants to light stress, we obtained from the SALK collection knock-out mutants for 70 genes encoding transcripts with altered

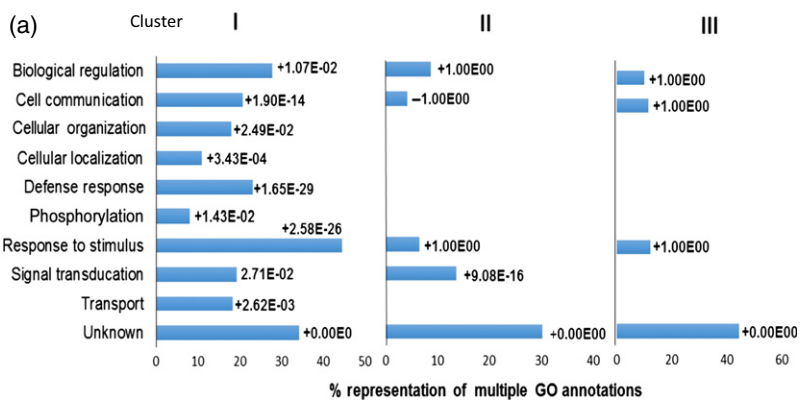
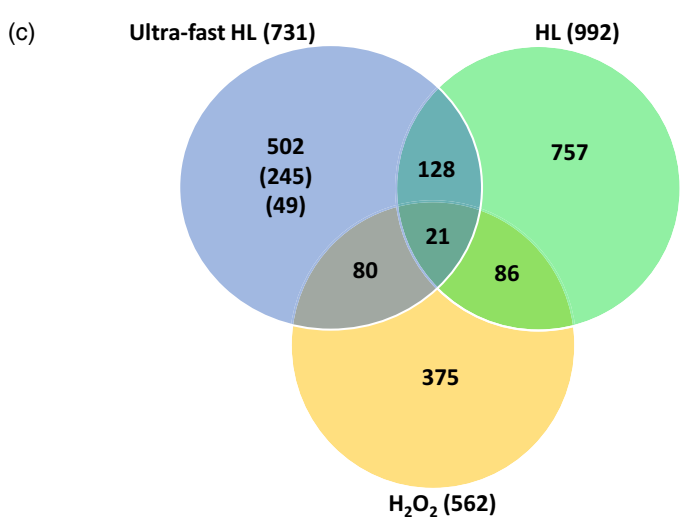


Figure 2. Meta-analysis of ultra-fast light stress-response transcripts in Arabidopsis.
(a) Gene Ontology annotation of the different transcripts found in the three ultra-fast response clusters shown in Figure 1(a).
(b) Distribution of abiotic and biotic stress-response transcripts between the different ultra-fast response clusters. Transcript representation higher than 10% is highlighted in bold.
(c) Venn diagram showing the overlap between ultra-fast response transcripts to light stress, transcripts accumulating in Arabidopsis following a 3-h light stress treatment and transcripts accumulating in Arabidopsis following a 1-h treatment with H_2O_2. Out of the 502 transcripts shown to be unique to ultra-fast high light (HL), 245 do not overlap with any of the abiotic stresses shown in (b). Out of those 245 transcripts, 49 do not appear on the ATH1 Affymetrix chips.

(b)

	Cluster I	Cluster II	Cluster III
Total	384	274	73
Abiotic stresses			
Drought	**59 (15 %)**	**83 (30 %)**	**21 (29 %)**
Cold	**120 (31 %)**	13 (5 %)	**19 (26 %)**
Heat	**59 (15 %)**	**59 (21 %)**	**34 (46 %)**
High light	**64 (17 %)**	**64 (23 %)**	**21 (29 %)**
NaCl	**116 (30 %)**	20 (7 %)	5 (7 %)
Ozone	**89 (23 %)**	5 (2 %)	4 (5 %)
Wounding	22 (6 %)	5 (2 %)	4 (5 %)
Incompatible bacterial pathogen	**43 (11 %)**	2 (0.7 %)	0



expression at 20 sec of light stress (O'Malley and Ecker, 2010) and screened them for tolerance to light stress. Seven knockouts showed enhanced cell death in response to light stress and a second independent knockout was obtained for these and screened again. As shown in Figure 3(a), five genes were found to be important for acclimation to light stress using two independent knockouts. They encoded two proteins of unknown function (At5g10695 and At3g10020), a Golgi-associated protein (At5g51430), a RAV transcription factor (At1g25560) and a glycosyl hydrolase (At3g13750), and were required to prevent light-induced cell death in Arabidopsis leaves. It should also be mentioned that a sixth protein identified by our analysis, Zat12 (Figure 1b), was previously shown to be required for acclimation to light stress (Iida *et al.*, 2000). Interestingly, as shown in Figure 3(b), none of the five transcripts functionally characterized in Figure 3(a) was induced in response to the 3-h light stress treatment (Kleine *et al.*, 2007). Also, none of the five transcripts shown in Figure 3(a) was found to be enhanced in a time-course experiment subjecting Arabidopsis to light stress as reported in Davletova *et al.* (2005a; Figures S2 and S3). The five ultra-fast transcripts assayed in Figure 3(a) were therefore primarily induced during early stages of the response of Arabidopsis to light stress (with two of them also induced by H_2O_2; Figure 3b). Our finding that these transcripts were primarily induced during the rapid response of plants to light stress, but not during later stages of this response (Figures 3b, S2 and S3; Davletova *et al.*, 2005a; Kleine *et al.*, 2007), highlight their potential biological role in protecting plants from light stress (Figure 3a), thus demonstrating that the rapid response is important for the tolerance of plants to light stress.

Transcriptional regulation of ultra-fast response transcripts

To determine if some of the ultra-fast transcripts identified by our analysis (Figure 1) were regulated, at least partially, at the transcriptional level, we used qPCR to test whether transcriptional inhibitors such as α-amanitin and actinomycin D would suppress their accumulation. We selected four different transcripts with a defined response pattern confirmed by qPCR for this analysis (Figure 4). As can be seen in Figure 4(a), changes in transcript accumulation, visualized by RNA-Seq read alignment maps, could be seen for the four selected transcripts across the entire length of their corresponding genes with some genes changing in expression two- to three-fold within 20 sec of light stress. Pre-treatment with α-amanitin or actinomycin D suppressed the accumulation of all four selected transcripts (Figure 4b), demonstrating that at least some of the transcripts accumulating at 20 sec of light stress could require active transcription for their regulation.

To further determine if the steady-state transcript level of some of the ultra-fast response transcripts is enhanced transcriptionally within seconds after light stress, we constructed different reporter genes in which the WRKY40 and the Zat12 promoters were fused to an unstable variant of the luciferase gene (Luc^u). As can be seen in Figure 5(a), subjecting three independent homozygous lines for each

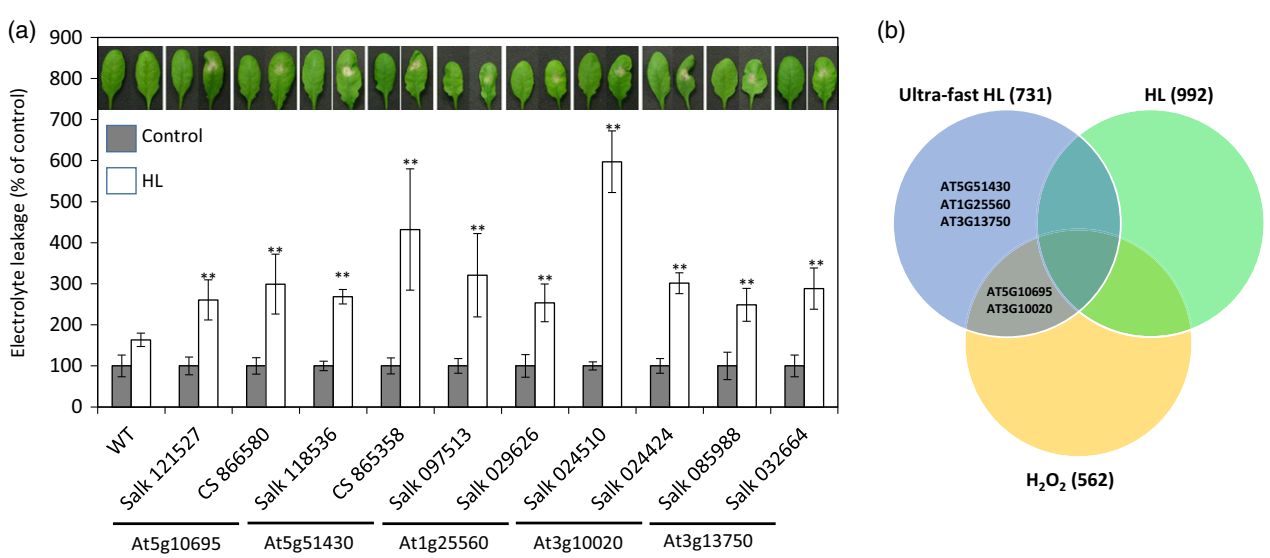


Figure 3. Functional analysis of selected ultra-fast response transcripts in Arabidopsis.
(a) Light stress-induced cell death in knockout mutants for five different genes encoding ultra-fast response transcripts. Two independent alleles for each gene were subjected to light stress and cell death was photographed and measured by electrolyte leakage. $^{**}P < 0.01$. HL, high light.
(b) Venn diagram showing the overlap between the five transcripts tested in (a), the ultra-fast response to light stress, the response of Arabidopsis to a 3-h light stress treatment and the response of Arabidopsis to a 1-h treatment with H_2O_2.

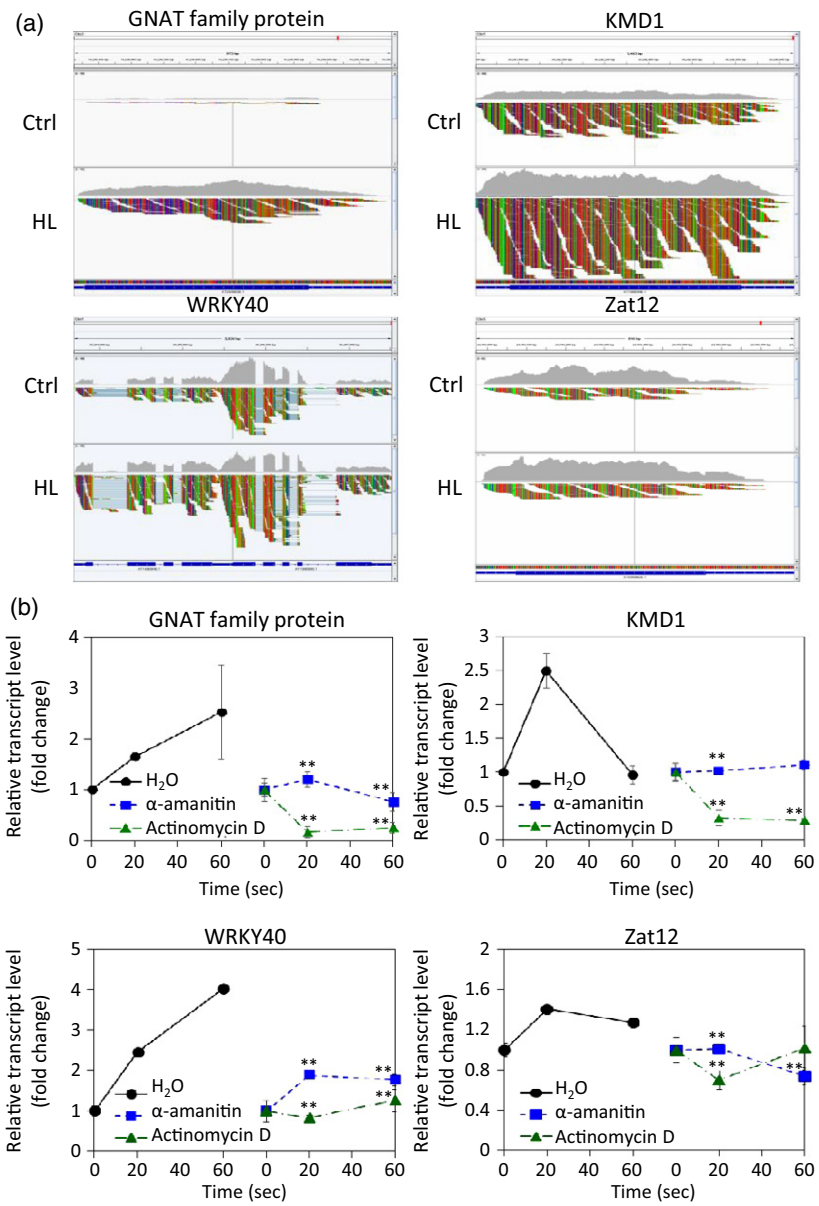


Figure 4. Suppression of light stress-induced ultra-fast accumulation of transcripts by transcriptional inhibitors.
(a) Standardized RNA sequencing read maps for four selected genes encoding ultra-fast response transcripts at 20 sec of light stress exposure. HL, high light.
(b) Transcript accumulation for the four genes shown in (a) measured by quantitative PCR in plants treated or untreated with two different transcriptional inhibitors (α-amanitin or actinomycin D) prior to light stress treatment. $^{**}P < 0.01$.

of these reporter genes to light stress for 20 sec resulted in enhanced luciferase activity that occurred within 40–60 sec in the Zat12::Luc^{u} and 110–140 sec in the WRKY40::Luc^{u} constructs. In addition, we used qPCR to measure the accumulation of the luciferase transcripts in Zat12::luc (Miller *et al.*, 2009; regular stable luciferase fused to the same Zat12 promoter fragment) plants 20 and 60 sec following application of light stress. As shown in Figure 5(b), luciferase transcripts driven by the Zat12 promoter could be detected as early as 20 sec following light stress. The findings shown in Figures 4(b) and 5 suggest that at least some of the transcripts with enhanced steady-state levels at 20 and 60 sec following application of light stress in Arabidopsis are driven by the activation of their promoters.

Because the steady-state level of many of the transcripts shown in Figure 1 could also be enhanced in response to light stress due to changes in their RNA stability (i.e. by increased stability during the early stages of light stress), we determined the content of 11 different RNA-destabilizing sequences (Ohme-Takagi *et al.*, 1993; Narsai *et al.*, 2007) in these transcripts and compared it with the content of the same RNA-destabilizing sequences in transcripts induced by light stress at 3 h (Kleine *et al.*, 2007). As shown in Figure S4, the content of the destabilizing sequences was very similar in these two transcript groups. However, further studies are needed to determine the role of RNA stability in the ultra-fast response of Arabidopsis to light stress.

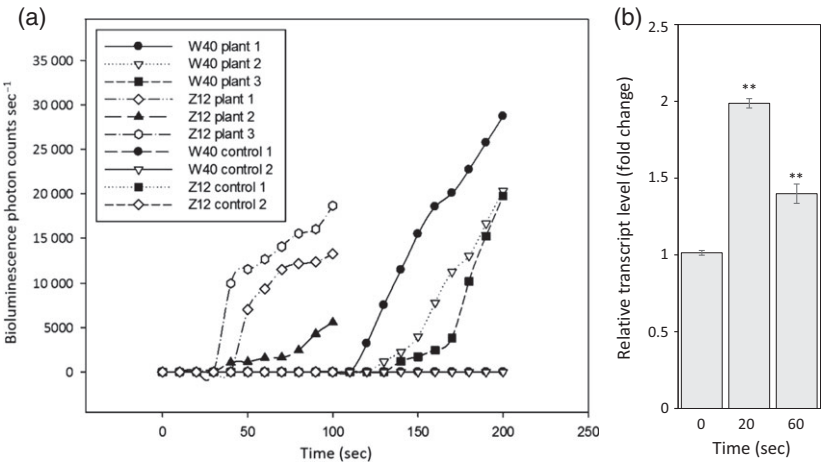


Figure 5. Analysis of the ultra-fast response to light stress using promoter::reporter constructs.
(a) Luciferase activity measurements of the ultra-fast light stress response using an unstable luciferase gene fused to the promoters of the Zat12 (measured from 0 to 100 sec) or WRKY40 (measured from 0 to 200 sec) genes. Three independent transgenic lines per construct are shown.
(b) Accumulation of the luciferase transcript, determined by quantitative PCR, during the early stages of light stress acclimation in Zat12::luc plants. $^{**}P < 0.01$.

Involvement of ABA and ROS in the ultra-fast response of Arabidopsis to light stress

Meta-analysis of the transcriptomic response at 20 and 60 sec of light stress revealed that 12–22% of the transcripts with an enhanced steady-state level in all clusters are ABA-response transcripts (Figure 6a; ATH1 chip data was obtained from the supplementary material of Nemhauser *et al.*, 2006; Blanco *et al.*, 2009; Davletova *et al.*, 2005b; Scarpeci *et al.*, 2008; Gadjev *et al.*, 2006). Twelve to 21% of transcripts from Cluster I were also brassinolide-, jasmonate- and H_2O_2-response transcripts. These findings suggest that Cluster I is distinct from Clusters II and III in its content of hormone-response transcripts (Figure 6a). To further test the dependence of the ultra-fast response on ABA signaling we studied the response of selected mutants impaired in ABA and retrograde signaling to light stress using the assay described in Figure 3(a). For this analysis we used *aba-1*, deficient in ABA biosynthesis, *abi-1*, deficient in ABA sensing via protein phosphatase 2C that is involved in NADPH oxidase activation in response to ABA, and *abi-4* and *gun-1*, deficient in retrograde signaling from the chloroplast to the nuclei (Koussevitzky *et al.*, 2007; Suzuki *et al.*, 2013a). As shown in Figure 6(b), of the different mutants tested only the *abi-1* mutant displayed enhanced tolerance to light stress, potentially due to an enhanced content of stress-response transcripts or deficiency in ABA-induced NADPH oxidase activation. Because protein phosphatase 2C (ABI-1) activity could be directly suppressed by enhanced H_2O_2 levels, or by enhanced ABA levels (through PYR/PYL; Mittler and Blumwald, 2015), the finding that the *abi-1* mutant is impaired in the response to light stress could suggest that this protein is involved, via H_2O_2 and/or ABA, in early light stress responses in Arabidopsis. Interestingly, as shown in Figure 6(c), a considerable overlap was found between the transcripts upregulated in untreated *abi-1* plants (Hoth *et al.*, 2002; data obtained from the supplementary material therein) and the transcripts enhanced in plants subjected to 3-h light stress (Kleine *et al.*, 2007) or the ultra-fast high light (HL) treatment described in this work. This finding could suggest that in Arabidopsis deficiency in ABI-1 mimics some aspects of the light stress response and that the *abi-1* mutant is more tolerant to light stress (Figure 6b) because it already activates some of these responses.

To further test the dependence of some ultra-fast response transcripts on H_2O_2/ROS we analyzed the expression of selected ultra-fast response transcripts in mutants impaired in ROS scavenging/signaling such as *apx1*, *cat2* and *rbohD*. *apx1* and *cat2* are impaired in H_2O_2 scavenging whereas *rbohD* is impaired ROS signaling (Torres *et al.*, 2002; Vanderauwera *et al.*, 2011). As can be seen in Figure 7, some of the transcripts tested were found to be dependent on ROS scavenging (WRKY40), some were independent of ROS signaling/scavenging (WRKY18 and KMD1) and some were only partially dependent on ROS scavenging (Zat12). Interestingly, the response of all transcripts tested was unaltered in the *rbohD* mutant, suggesting that RBOHD may not be involved in the regulation of these transcripts and that the possible function of ABI1 in this response could be different from its function in classical ABA sensing. Further studies are of course required to unravel the role of ROS and ABA in the ultra-fast response of plants to light stress.

DISCUSSION

Several lines of evidence suggest that the ultra-fast transcriptome response reported here is biologically-important. It was composed of 731 transcripts that demonstrated an ordered and clustered response (Figure 1, Tables S1–S3 and S5–S7); it included several ultra-fast response-specific transcripts that were required for acclimation to light stress (Figure 3; Iida *et al.*, 2000); the different clusters included in the response were composed of transcripts with differential responsiveness to different abiotic/biotic stresses,

(a)

	Cluster I	Cluster II	Cluster III
Total	385	274	73
Hormone/ROS			
ABA	**47 (12.21)**	**62 (22.63)**	**14 (19.18)**
ACC	7 (1.82)	7 (2.55)	0 (0.00)
Brassinolide	**47 (12.21)**	5 (1.82)	0 (0.00)
Cytokinin	9 (2.34)	3 (1.09)	1 (1.37)
Gibberellin	3 (0.78)	0 (0.00)	1 (1.37)
Indole-3-acetic acid	34 (8.83)	18 (6.57)	0 (0.00)
Methyl jasmonate	**49 (12.73)**	16 (5.84)	7 (9.59)
SA	29 (7.53)	1 (0.36)	0 (0.00)
H_2O_2	**83 (21.56)**	10 (3.65)	**8 (10.96)**
O_2^-	14 (3.64)	1 (0.36)	4 (5.48)
$^1O_2$	13 (3.38)	4 (1.46)	2 (2.74)

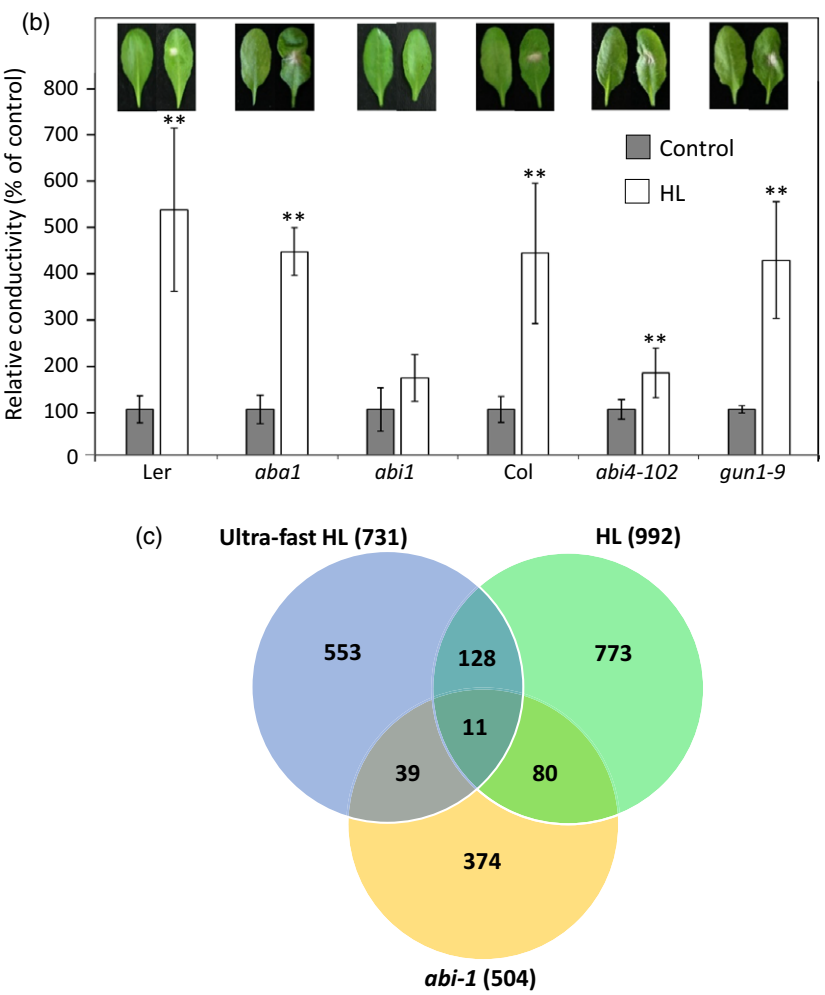


Figure 6. Distribution of hormone- and reactive oxygen species (ROS)-response transcripts between the different ultra-fast response clusters and tolerance of different mutants impaired in ABA and retrograde signaling to light stress.
(a) Meta-analysis of the different ultra-fast clusters showing that Cluster I is distinct from Clusters II and III in its content of hormone-response transcripts. Transcript representation higher than 10% is highlighted in bold. ACC, ethylene; SA, salicylic acid.
(b) Light stress-induced cell death in different mutants impaired in ABA and retrograde signaling. $^{**}P < 0.01$. HL, high light.
(c) Venn diagram showing the overlap between transcripts accumulating in the *abi-1* mutant in the absence of stress, ultra-fast response transcripts to light stress and transcripts accumulating in Arabidopsis following a 3-h treatment of light stress.

ROS or hormones (Figures 2 and 6); expression of some of the transcripts involved in the response could be suppressed by transcriptional inhibitors (Figure 4); and it included transcripts that responded differentially when studied in a genetic background that was altered in ROS scavenging responses (Figure 7). Taken together, these observations indicate that very rapid responses at the mRNA level in plants could play an important biological role in the acclimation of plants to stress.

It is possible that many of the ultra-fast changes observed in the steady-state transcript level in response to light stress in our study (Figure 1a, b, Tables S1–S3 and

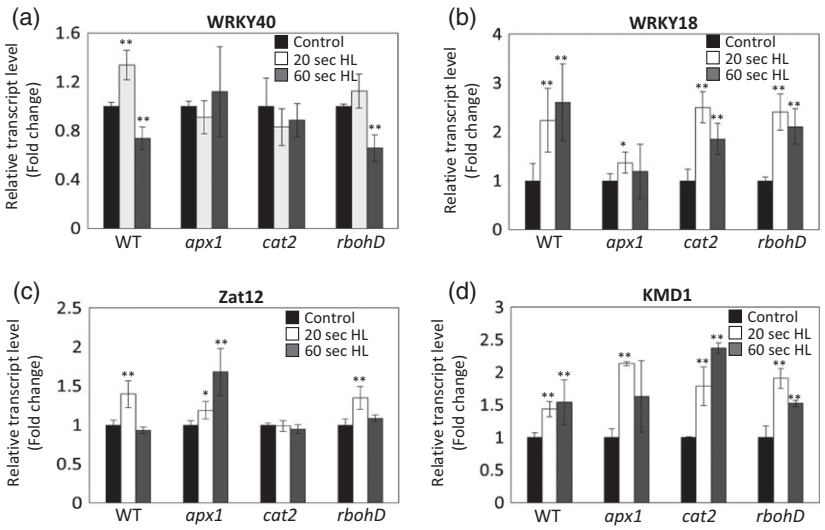


Figure 7. Expression of four selected ultra-fast transcripts in two genetic backgrounds impaired in reactive oxygen species (ROS) scavenging (*apx1* and *cat2*) and in a genetic background impaired in ROS signaling (*rbohD*).
(a) WRKY40.
(b) WRKY18.
(c) Zat12.
(d) KMD1.
$*P < 0.05$; $**P < 0.01$.

S5–S7) were caused by post-transcriptional alterations in RNA stability. Furthermore, many of the ultra-fast response transcripts contained RNA-destabilizing sequences, albeit not at a proportion higher than late (3 h) light stress-response transcripts (Figure S4). Nevertheless, the findings that transcriptional inhibitors suppressed the expression of some ultra-fast response transcripts (Figure 4), and that the ultra-fast response could be observed via promoter::reporter constructs (Figure 5), combined with several reports of stalled RNA polymerases found on the promoters of eukaryotic genes (Nechaev and Adelman, 2008; Levine, 2011; Kwak and Lis, 2013), strongly suggest that some of the ultra-fast response transcripts reported here are regulated at the transcriptional level and could contain stalled but active RNA polymerases at their promoters. This possibility, combined with reported transcription rates of over 50 kb min^{-1} in eukaryotic cells (Maiuri *et al.*, 2011), could explain the rapid accumulation of some of the transcripts observed in our experiments (Figures 1–4, Tables S1–S3), especially considering the relatively short length of Arabidopsis genes (average of 2000 bp; Arabidopsis Genome Initiative, 2000). Differential RNA stability and stalled RNA polymerases could therefore provide a mechanistic explanation for the ultra-fast transcriptome reprograming observed in response to light stress in Arabidopsis.

In contrast to the rapid accumulation of different transcripts, the ultra-fast transcriptome response of Arabidopsis to light stress included a rapid decline in the level of many transcripts (Figure S1a, Tables S6 and S7). Genome-wide analyses of decline of mRNA in Arabidopsis have revealed three different groups of transcripts that are prone to rapid mRNA decline: transcripts encoded by intronless genes, transcripts possessing destabilizing sequences in their 3′ end and transcripts that are targets of microRNA regulation (Narsai *et al.*, 2007). In addition, uncapping-mediated mRNA degradation was found to be associated with abiotic stress responses (Zhang *et al.*, 2013). Nevertheless, the majority of studies focusing on mRNA decline in plants did not use short time points such as 20 and 60 sec and further analysis is needed to decipher the mechanisms that regulate mRNA decline under the conditions described in this study.

Taking into account the 'harsh' biological assay we used to define a function in light stress acclimation (i.e. cell death; Figure 3), it is likely that many other transcripts identified by our transcriptome analysis could play a role in light stress acclimation, albeit less significant than prevention of cell death. The relationship between ROS and the expression of many of the genes identified by our analysis is unclear at present. High levels of ROS did not accumulate in cells during the first 0–90 sec of light stress (Figure 1b), but the expression of some ultra-fast response genes was dependent on ROS signaling (Figure 7). This finding suggests that the high antioxidant capacity of plant cells is able to handle the initial rise in ROS produced during early stages of light stress (Figure 1c), but that several genes that are highly sensitive to ROS levels still react (Figures 1b and 7). In addition to these, proteins inhibited by H_2O_2, such as ABI-1, could also be affected by low levels of ROS (Figure 6b, c). The rapid APX1-dependent depletion of ascorbic acid levels in response to light stress (Figure 1c) supports the hypothesis that plants have a high buffering capacity for rapid changes in ROS levels. Key players in the regulation of rapid transcriptional responses could therefore be localized and/or low-level ROS signals, calcium signatures or ABI-1 and/or conjugated ABA (Miller *et al.*, 2009; Mittler *et al.*, 2011, 2012; Suzuki *et al.*, 2013a; Choi *et al.*, 2014; Gilroy *et al.*, 2014; Mittler and Blumwald, 2015).

Abscisic acid was shown to play a key role in the response of plants to abiotic stresses, and ABA and ROS were shown to coordinate responses to the ROS wave in

systemic tissues (Suzuki *et al.*, 2013a; Mittler and Blumwald, 2015). Our findings that many of the ultra-fast response transcripts are also ABA-response transcripts (Figure 6a) and that the *abi-1* mutant has an impaired response to light stress (Figure 6b) is therefore in line with the potential role of ABA in this response. Because, in addition to its function in the ABA pathway, the ABI-1 protein could also be directly inhibited by H_2O_2 (Mittler and Blumwald, 2015), an alternative explanation for our findings could also be that during the ultra-fast response to light stress the little H_2O_2 that is accumulated is directly inhibiting this protein. In this case the deficiency in ABI-1 protein in the *abi-1* mutant will mimic the response of these plants to light stress and pre-condition them to resist light (Figure 6b, c). Further studies are required to address these possibilities.

Virtually all time-course omics studies of plant or animal responses to abiotic stress lack early time points (seconds to minutes; e.g. Hruz *et al.*, 2008). Our findings demonstrate that during these early stages of the stress response the cell undergoes a reprogramming of its transcriptome that could affect the activation of signal transduction pathways and the establishment of successful acclimation. The large number of transcripts that respond to light stress within 20–60 sec in Arabidopsis, uncovered by our analysis (Figure 1, Tables S1–S3 and S5–S7), combined with the important role that some of these early response transcripts play in light stress tolerance (Figure 3; Iida *et al.*, 2000), highlight the importance of these early stages for plant acclimation. Further characterization of these transcripts, as well as additional studies of the ultra-fast response of plants to other abiotic and biotic stresses, could lead to the development of new and novel approaches to enhance the tolerance of plants and crops to different environmental stresses using pathways and compounds that were not previously known, or considered to be involved in abiotic stress.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana Col-0 (cv. Columbia-0), WS-0 (cv. Wassilewskija-0), L*er*-0 (cv. Landsberg *erecta*), *rbohD*, *apx1*, *aba1*, *abi1*, antisense Cat2 (Torres *et al.*, 2002; Davletova *et al.*, 2005a; Koussevitzky *et al.*, 2007; Vanderauwera *et al.*, 2011; Suzuki *et al.*, 2013a, b) and confirmed knockout lines for 70 genes encoding high-light-response transcripts (O'Malley and Ecker, 2010; Table S1) were grown in peat pellets (Jiffy-7, Jiffy, http://www.jiffygroup.com/en/) or soil mixture (MetroMix 200, SUN GRO, http://www.sungro.com/) in 9 x 9 x 6 cm^3 pots covered with a fiberglass screen net at 23°C under constant low light (50 μmol m^{-2} sec^{-1}). Knockout lines were obtained from ABRC (http://abrc.osu.edu/) and bulked together with wild-type seeds under carefully controlled growth conditions as previously described (Suzuki *et al.*, 2011; Luhua *et al.*, 2013). Zat12::luc plants were obtained as described in Miller *et al.* (2009). A similar strategy was also used to fuse the promoters of Zat12 (Miller *et al.*, 2009) and WRKY40 (1.2 kb), to the unstable form of Luciferase [Promega, http://www.promega.com/; pGL4.11(luc2p), designated here as Luc^U] (Davletova *et al.*, 2005b; Miller *et al.*, 2009). Luciferase activity was imaged as described by Miller *et al.* (2009). One leaf of 16- to 20-day-old plants was sprayed with 1 mM luciferin (GOLD Biotechnology, https://www.goldbio.com/), exposed to light stress (1500 μmol m^{-2} sec^{-1}) using a goose neck bulb, and imaged using a NightOWL LB983 NC100 (Berthold, https://www.berthold.com/) imager. Images were captured every 10 sec for 300 sec. Bioluminescence in photon counts sec^{-1} was measured using INDIGO v.2.0.3.0 (Berthold). Treated samples were compared to their respective controls and graphed.

Light stress treatment

For RNA-Seq, qPCR analyses and measurements of H_2O_2 and ascorbic acid, 15–20 plants grown in pots for 18–21 days as described above were exposed to a light intensity of 1000 μmol m^{-2} sec^{-1} at 21°C for periods of 0, 20 and 60 sec, 0, 10, 60 and 300 sec, or 0, 20, 30, 60 and 90 sec in a growth chamber (E-30-HB, Percival Scientific, http://www.percival-scientific.com/). Samples were collected by immediately dipping the pots in liquid nitrogen. Frozen tissues were then cut onto aluminum foil, ground and transferred into 1.5-ml tubes (about 100–150 mg per tube). Samples were kept frozen during the entire collecting process and stored at −80°C. For treatment with transcriptional inhibitors, plants grown in pots were sprayed with water, 10 μM α-amanitin or 75 μg ml^{-1} actinomycin D and incubated for 90 min at room temperature (21°C) prior to exposure to light stress for periods of 0, 20 and 60 sec. To test the tolerance of plants to light stress, one fully expanded rosette leaf of 21- to 25-day-old plants grown on peat pellets was exposed to 2000 μmol m^{-2} sec^{-1} high light for 1 h using a gooseneck light source (ACE I; Schott, http://www.amscope.com/). Leaves were then photographed and analyzed for electrolyte leakage as described below.

RNA-Seq, qRT-PCR, meta-analyses and ascorbic acid measurements

For RNA-Seq analysis, three independent biological replicates, each composed of leaves pooled from at least 30 different plants in three technical repeats, were used for each experimental condition. Total RNA was isolated and purified as described in Suzuki *et al.* (2013a) and RNA-Seq analysis was conducted using an Illumina HiSeq2000 at the University of Wisconsin-Madison Biotechnology Gene Expression Center. Gene Ontology annotations of the transcripts identified by our RNA-seq analyses were performed using PANTHER (http://www.pantherdb.org/pathway/) or obtained from TAIR (https://www.arabidopsis.org/tools/bulk/go/index.jsp). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described (Miller *et al.*, 2009; Suzuki *et al.*, 2013a) using a StepOnePlusTM Real-Time PCR System (Applied Biosystems, http://www.appliedbiosystems.com/). The qPCR data were analyzed with STEPONEPLUS software v.2.0.1 (Applied Biosystems). Threshold cycle values for Zat12, WRKY18, WRKY40, APX1, RbohD, GNAT5 and KMD1 were calculated with the CT of EF1-a as an internal control. Primer pairs used for amplifications are shown in Table S9. The overlap between transcripts enhanced in leaves in response to short-term high-light exposure and transcripts enhanced in response to ABA, ethylene (ACC), brassinolide (BL), cytokinin (CK), gibberellin (GA), auxin (IAA), methyl jasmonate (MJ), salicylic acid (SA), H_2O_2, O_2^- or $^1O_2$ (Davletova *et al.*, 2005b; Gadjev *et al.*, 2006; Nemhauser *et al.*, 2006; Scarpeci *et al.*, 2008; Blanco *et al.*, 2009), or in response to differ-

ent abiotic stresses (Tosti *et al.*, 2006; Truman *et al.*, 2006; Kleine *et al.*, 2007; Huang *et al.*, 2008; Larkindale and Vierling, 2008; Matsui *et al.*, 2008; Consales *et al.*, 2012; Ding *et al.*, 2014), was determined as previously described (Miller *et al.*, 2009; Suzuki *et al.*, 2013a). Levels of ascorbic acid were measured by gas chromatography–mass spectrometry (GC-MS) analysis and expressed as relative to an internal control (ribitol) as previously described (Rizhsky *et al.*, 2004; Suzuki *et al.*, 2013a).

H_2O_2 measurement

The accumulation of H_2O_2 in tissues exposed to the short-term light stress was measured using Amplex Red (Molecular Probes, Invitrogen, http://www.invitrogen.com/). Five hundred microliters of 50-mM sodium phosphate buffer (pH 7.4) containing 50 μM Amplex Red and 0.05 U ml^{-1} horseradish peroxidase was added to ground tissues and samples were centrifuged at 12 000 *g* for 12 min at 4°C. Following the centrifugation, 450 μl of supernatant was transferred into fresh tubes and incubated for 30 min at room temperature in the dark. Absorbance at 560 nm was then measured and the concentration of H_2O_2 in each sample was determined from a standard curve consisting of 0, 0.5, 1, 3, 6 and 9 μM of H_2O_2. Following the measurement of absorbance, tissue samples were completely dried using a speed vacuum concentrator at 30°C for 30 min and H_2O_2 accumulation per mg dry weight was calculated.

Electrolyte leakage assay

Electrolyte leakage was measured as described by Sung and Guy (2003) with minor modifications. Briefly, one fully expanded leaf exposed to high light as described above was excised and immersed in 10 ml of distilled, deionized water in a 50-ml Falcon tube. Samples were shaken for 1 h at room temperature and the conductivity of the water was measured using a conductivity meter (Sung and Guy, 2003). Samples were then heated to 95°C for 20 min using a heat block, shaken for 1 h at room temperature and the conductivity of the water was measured again. The percentage of electrolyte leakage was calculated as the percentage of the conductivity before heating over that after heating.

Statistical analysis

We performed next generation RNA-Seq for differential expression profiling and characterization of transcript processing events. Three biological replicates were obtained as described above. Paired-end Illumina sequencing generated on average 21 million read pairs per sample, with each sequence read having a length of 101 nucleotides. We utilized the services of frequently used, publicly available RNA-Seq analysis software, namely BOWTIE (Langmead *et al.*, 2009), TOPHAT (Trapnell *et al.*, 2009) and CUFFLINKS (Trapnell *et al.*, 2010), for alignment of paired-end reads onto the reference genome, parsing the alignment to infer the exon–exon splice junctions, and performing the differential expression analysis of annotated genes. Transcripts expressing differentially in two (or more) conditions were identified by examining the difference in their abundance under the two conditions. The abundance of a transcript is measured in terms of FPKM, normalized for the transcript length and total number of cDNA fragments for a sample replicate. The difference in expression was obtained as the log of fold change in abundance between the two conditions. A test of statistical significance for differential expression of each transcript was performed based on a negative binomial model estimated from the data (Trapnell *et al.*, 2010). The fold change of genes with multiple isoforms was assessed by summing up the FPKMs for all isoforms of a gene and then measuring the difference between the two conditions (Trapnell *et al.*, 2010). Other statistical analyses were performed by one-tailed Student's *t*-test as previously described in Suzuki *et al.* (2013a) . Results are presented as mean ± SD (*$P < 0.05$; **$P < 0.01$).

We extracted three distinctive patterns representing elevation in steady-state transcript level at 20 or 60 sec or both using a Fortran code on a Unix platform: Cluster I, elevation of steady-state transcript level at 20 sec followed by decline at 60 sec; Cluster II, elevation of steady-state transcript level at 60 sec with no significant change at 20 sec; and Cluster III, elevation of steady-state transcript level at 20 sec and 60 sec. Elevation or decline in steady-state transcript level at a particular time point indicated a statistically significant increase or decrease, respectively, in steady-state transcript level relative to the previous time point. Steady-state transcript level refers to transcript abundance which is measured in terms of FPKM, normalized for the transcript length and total number of cDNA fragments for a replicate sample.

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AUTHOR CONTRIBUTIONS

NS, RM, GM, VS, RA designed and supervised the research. NS, ES, RA, LS, AB and AD performed the research and analyzed the data. RM and NS wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. (a) Histogram showing the fold change distribution of all transcripts that had a significant change in their steady-state transcript level (increase or decrease) in response to light stress.

Figure S1. (b) Distribution of genes encoding transcripts up-regulated at 20 sec of light stress on all Arabidopsis chromosomes.

Figure S2. Changes in the steady-state transcript level of the five genes tested with mutants in Figure 3 in response to light stress.

Figure S3. Heat map showing changes in the steady-state transcript level of the 682 transcripts that appear in the ATH1 Affymetrix chips (out of 731 from Clusters I, II and II; Figure 1) in response to light stress.

Figure S4. Content of RNA-destabilizing sequences in ultra-fast response transcripts and transcripts responding to a 3 h light stress treatment.

Table S1. Transcripts with a significant enhancement in steady state transcript level at 20-sec high-light exposure (Cluster I).

Table S2. Transcripts with a significant enhancement in steady state transcript level at 60-sec high-light exposure (Cluster II).

Table S3. Transcripts with a significant enhancement in steady state transcript level at 20- and 60-sec high-light exposure (Cluster III).

Table S4. Transcripts in Clusters I, II and III, not found in Affymetrix ATH1 chips.

Table S5. Transcripts in Clusters I, II and III divided into those enhanced from less than a 2.0 FPKM value and from more than a 2.0 FPKM value.

Table S6. Transcripts with a significantly declined steady-state transcript level at 20-sec high-light exposure.

Table S7. Transcripts with a significantly declined steady-state transcript level at 60-sec high-light exposure.

Table S8. Transcripts in Clusters I, II and III not found to be enhanced by a longer (3 h) light stress treatment or any of the other abiotic stresses tested (Figure 2b).

Table S9. Primer pairs used for quantitative real-time PCR.

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