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Original Article

Blocking the Q_B-binding site of photosystem II by tenuazonic acid, a non–host-specific toxin of *Alternaria alternata*, activates singlet oxygen-mediated and EXECUTER-dependent signalling in *Arabidopsis*

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ABSTRACT

Necrotrophic fungal pathogens produce toxic compounds that induce cell death in infected plants. Often, the primary targets of these toxins and the way a plant responds to them are not known. In the present work, the effect of tenuazonic acid (TeA), a non–host-specific toxin of *Alternaria alternata*, on *Arabidopsis thaliana* has been analysed. TeA blocks the Q_B-binding site at the acceptor side of photosystem II (PSII). As a result, charge recombination at the reaction centre (RC) of PSII is expected to enhance the formation of the excited triplet state of the RC chlorophyll that promotes generation of singlet oxygen ($^1O_2$). $^1O_2$ activates a signalling pathway that depends on the two EXECUTER (EX) proteins EX1 and EX2 and triggers a programmed cell death response. In seedlings treated with TeA at half-inhibition concentration $^1O_2$-mediated and EX-dependent signalling is activated as indicated by the rapid and transient up-regulation of $^1O_2$-responsive genes in wild type, and its suppression in *ex1/ex2* mutants. Lesion formation occurs when seedlings are exposed to higher concentrations of TeA for a longer period of time. Under these conditions, the programmed cell death response triggered by $^1O_2$-mediated and EX-dependent signalling is superimposed by other events that also contribute to lesion formation.

Key-words: necrotrophic pathogens; programmed cell death; Arabidopsis thaliana; photosynthetic electron transport.

INTRODUCTION

Based on different strategies used to colonize plants, fungal pathogens can be divided into biotrophs and necrotrophs. Biotrophic fungal pathogens need to establish an intimate interaction with living host cells for feeding, while necrotrophs acquire nutrients from dead cells killed prior to or during colonization of their host (Mengiste 2012).

Necrotrophic fungal pathogens produce diverse secondary metabolites and peptides that promote cell death of infected plants and thus have been considered to be toxins (Lou *et al.* 2013). These toxins may be host-specific or non–host-specific (Mengiste 2012; Stergiopoulos *et al.* 2012; Tsuge *et al.* 2013). Host-specific toxins affect single plant species or particular genotypes of a given species and are responsible for the pathogenicity and virulence of the fungus, whereas non–host-specific toxins are not a main determinant of pathogenicity, but contribute to it and are effective on many plant species (Mengiste 2012). Isolates of a given necrotrophic fungal pathogen species may produce different, sometimes isolate-specific host- and non–host-specific toxins, suggesting that during evolution, these fungi have successfully developed highly variable tools to colonize a wide range of plants (Thomma 2003; Stergiopoulos *et al.* 2012; Lou *et al.* 2013; Tsuge *et al.* 2013). In order to understand the evolutionary forces that allowed necrotrophic fungi to expand their host range, one need to analyse the mode of action of particular toxins and the way a potential host plant reacts to them (McDonald *et al.* 2013). In the present work, the effect of tenuazonic acid (TeA), a non–host-specific toxin of *Alternaria alternata* (Thomma 2003), on *Arabidopsis thaliana* was analysed to identify mechanisms that may help a necrotrophic pathogen to colonize its host plant.

TeA blocks photosynthetic electron transport at the acceptor side of photosystem II (PSII) by competing with plastoquinone B (Q_B) for the Q_B-binding site of the reaction centre (RC) protein D1 of PSII (Chen *et al.* 2007). As a result of this block of the electron transport chain, the reduced form of plastoquinone A (Q_A) starts to accumulate and charge recombination at the RC of PSII takes place that gives rise to the formation of the triplet state of the excited chlorophyll P680. This excited form of chlorophyll may act as a photosensitizer and transforms by energy transfer the ground state triplet oxygen into the highly reactive singlet oxygen ($^1O_2$) (Foote 1968; Vass & Cser 2009).

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Formation of $^1O_2$ in chloroplasts may lead to various stress responses of plants (op den Camp *et al.* 2003). At higher concentrations, $^1O_2$ causes oxidative damage by directly interacting with molecules such as proteins, polyunsaturated fatty acids and DNA and in this way irreversibly destroy or alter the function of these targets (Triantaphylidès & Havaux 2009). Some of the oxidation products are biologically active and may act as signalling molecules (Mueller *et al.* 2008; Ramel *et al.* 2012). Under less severe stress, $^1O_2$ may initiate signalling in the absence of oxidative damage (Kim *et al.* 2012). In such cases, $^1O_2$ signalling depends on the activity of the plastid proteins EXECUTER1 (EX1) and EXECUTER2 (EX2) (Wagner *et al.* 2004; Lee *et al.* 2007). Growth inhibition, cell death and lesion formation triggered by $^1O_2$-mediated and EX-dependent signalling are abrogated in an *executer1 (ex1)/executer2 (ex2)* mutant of *A. thaliana*, even though these mutant plants release similar amounts of $^1O_2$ as *EX1/EX2* control plants (Kim *et al.* 2012). To understand TeA's mode of action during colonization of host plants by necrotrophic fungal pathogens, we have asked whether TeA, by targeting the photosynthetic electron transport, activates $^1O_2$-mediated and EX-dependent signalling and triggers a cell death response in toxin-treated *Arabidopsis* plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild type, the *ex1ex2* double mutant, the *flu* mutant and the *flu* mutant expressing the *SSU–GFP* transgene under control of the cauliflower mosaic virus 35S promoter, all in a Columbia-0 background, have been described previously (Kim *et al.* 2012). Seeds were surface-sterilized for 3 min using 2% bleach and then washed 10 times with sterile distilled H_2O. After sterilization, seeds were placed on 0.7% agar plates (100 × 25 mm) containing 1/2 Murashige-Skoog (MS) medium and 1 × Gamborg vitamins without sucrose and kept in the dark at 4 °C for 2 d. Seeds were then transferred to moderate light (90 μmol photons m^{-2} s^{-1}) and room temperature (22 °C) and seedlings were grown for 5 d under continuous light.

TeA treatment

TeA was isolated and purified from a culture of the *A. alternata* isolate 501 that belongs to *A. alternata* (Fr.) Keissler (Chen *et al.* 2007). It has been isolated from the Crofton weed (*Ageratina adenophora*), an invasive species in China, and causes the leaf brown spot disease (Qiang *et al.* 1999). For all experiments, TeA was initially dissolved in 100% dimethyl sulphoxide (DMSO) and was then diluted with sterile distilled H_2O to a final concentration of 0.1%. Five-day-old wild type and *ex1/ex2* mutant seedlings were transferred from continuous light to the dark, and after 30 min were sprayed either with 0.1% DMSO (mock) or different concentrations of TeA (250 μM, 500 μM, 1 mM, 2 mM) and kept in the dark for another 30 min. Depending on the experiment, seedlings were either transferred to continuous moderate light for 3 and 6 h or 1, 2 and 3 d or were kept in the dark for another 3 or 6 h after the TeA treatment.

Chlorophyll *a* fluorescence measurements

Five-day-old seedlings were treated with different concentrations of TeA for 3 h and 1–3 d, respectively. Chlorophyll *a* fluorescence transient (OJIP) curves of seedlings were measured at room temperature with a plant efficiency analyser (Handy PEA fluorometer, Hansatech Instruments, Ltd., King's Lynn, Norfolk, UK) as described by Strasser & Govindjee (1992). Before each measurement, seedlings were dark-adapted for 30 min. Chlorophyll *a* fluorescence was induced by 1 s pulses of red light (650 nm, 3500 μmol photons m^{-2} s^{-1}). The JIP test was used to analyse each chlorophyll *a* fluorescence OJIP transient (Strasser *et al.* 2004). The initial fluorescence F_o was measured at 20 μs; at this time all RCs are open after dark adaptation. The fluorescence intensities F_j and F_i were taken at 2 ms (J step) and 30 ms (I step), respectively; the maximal fluorescence intensity F_m was equal to F_p as the pulse was saturating. The JIP test defines the maximal (subscript 'o') energy fluxes in the energy cascade for the events 'absorption' (ABS), 'trapping' (TR) and 'electron transport' (ET). The relative variable fluorescence V_t is defined as $(F_t - F_o)/(F_m - F_o)$; the difference kinetics ΔV is defined as V_t (treated) – V_t (control); thus, the variable fluorescence at the 'J' time point (V_j) is defined as $(F_j - F_o)/(F_m - F_o)$. The maximum quantum yield of PSII primary photochemistry, PHI(P_0), is defined as TR_0/ABS = $1 - (F_o/F_m) = F_v/F_m$. The probability that an absorbed photon moves an electron further than Q_A is defined as $PHI(E_0) = ET_0/TR_0 = (1 - V_j)$. The performance index PI_{ABS} is used to express overall photosynthetic efficiency, which is calculated based on absorption as described by Strasser *et al.* (2004).

Measurements of cell death and chloroplast leakage

The percentage of seedlings with lesions was determined after 3 d of TeA treatment of 5-day-old wild type and *ex1/ex2* seedlings and the subcellular distribution of green fluorescent protein (GFP) by confocal scanning microscopy was done as described previously (Kim *et al.* 2012).

RNA extraction and RT-PCR

Five-day-old wild-type and *ex1/ex2* seedlings were incubated with 250 μM TeA for 3 and 6 h in the dark or under moderate light (90 μmol photons m^{-2} s^{-1}). Five to six seedlings were taken and immediately frozen with liquid nitrogen. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 0.7 μg RNA, treated with DNase (Promega, Madison, WI, USA) by utilizing Random Primers (Promega) and Improm II reverse transcriptase (Promega) according to the kit's protocol.

For RT-PCR, the samples were pre-heated at 95 °C for 5 min; the cycling conditions were: 20 s at 95 °C, 20 s at 60 °C, and 50 s 72 °C; 30 cycles. Primers used for RT-PCR are listed in Supporting Information Table S4.

Illumina library preparation

For the preparation of the Illumina libraries total RNA was extracted either from 5-day-old light-grown wild-type and *flu* mutant seedlings transferred to the dark for 8 h and re-exposed to light for various lengths of time or from 5-day-old wild-type and *ex1/ex2* mutant seedlings exposed for up to 24 h to the combined higher light/lower temperature stress (Kim *et al*. 2012). To assess the quality of RNA samples the concentration, integrity, and contamination of ribosomal RNA were checked using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Barcoded cDNA libraries from five micrograms of total RNA were prepared for multiplex sequencing on the Illumina HiSeq2000 platform (HiSeq 2000 User Guide (15011190U)) using a modified TruSeq method to construct strand-specific RNA-seq libraries (Zhong *et al*. 2011). The libraries were sequenced on the Illumina HiSeq2000 system at the Cornell University Life Sciences Core Laboratories Center.

Sequence processing and analysis

The raw Illumina RNA-seq reads were processed to remove low-quality regions and adaptor sequences using an in-house perl script. RNA-Seq reads were first aligned to ribosomal RNA and tRNA sequences by Bowtie (Langmead *et al*. 2009) to remove any possible contaminations of these sequences. The resulting filtered reads were aligned to the *Arabidopsis* TAIR10 genome using TopHat (Trapnell *et al*. 2009). Following the alignments, raw counts for each *Arabidopsis* gene were normalized to reads per kilobase of exon model per million mapped reads (RPKM) (Van Verk *et al*. 2013). Differentially expressed genes were identified using DESeq (Anders & Huber 2010). Raw *P*-values of multiple tests were corrected using a false discovery rate (Benjamini & Hochberg 1995).

RESULTS

Fast chlorophyll *a* fluorescence rise transients in *Arabidopsis* wild type and *ex1/ex2* seedlings treated with TeA

TeA has been shown to inhibit photosynthetic electron transport beyond Q_A on the acceptor side of PSII (Chen *et al*. 2007). In this way, its activity resembles closely that of other PSII inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and atrazine that also interrupt the electron flow between Q_A and Q_B (Przibilla *et al*. 1991; Oettmeier *et al*. 2001). The analysis of chlorophyll fluorescence kinetics in TeA-treated wild type and *ex1/ex2* mutant seedlings of *Arabidopsis* reveals that the primary toxin effect on photosynthetic electron transport does not seem to be altered by the lack of EX1 and EX2 activities in chloroplasts. The fast chlorophyll fluorescence induction shows a polyphasic OJIP transient with 'O' marking the original point of fluorescence and 'P' the fluorescence peak (Strasser & Govindjee 1992). Stress or treatment with TeA results in a strong increase of the 'J' level relative to the other components of the chlorophyll fluorescence change, which has been taken as evidence for an accumulation of reduced Q_A because of a slowdown of electron transport beyond Q_A (Strasser *et al*. 2004; Chen *et al*. 2007).

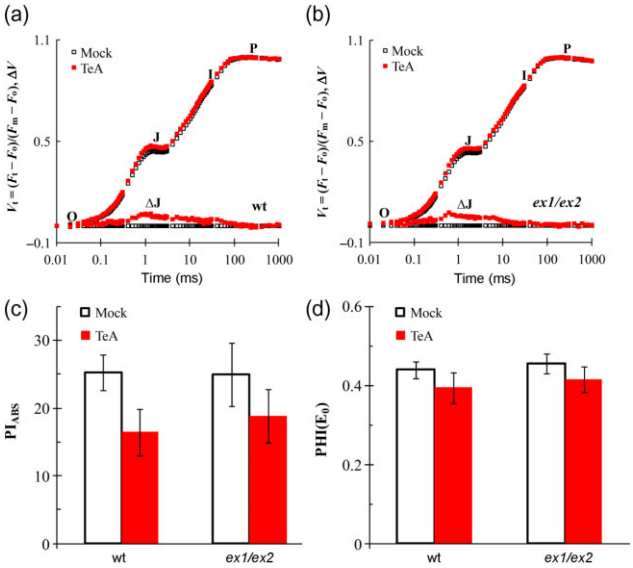

Figure 1. Chlorophyll fluorescence induction kinetics of (a) wild type (wt) and (b) *ex1/ex2* seedlings of *Arabidopsis thaliana* treated with TeA or 0.1% dimethyl sulphoxide (DMSO) (mock). The upper curves of (a) and (b) represent the relative variable fluorescence rise normalized between F_o and F_m and plotted as V_t changes on a logarithmic scale[$V_t = (F_t - F_o)/(F_m - F_o)$]. From these OJIP curves (Strasser & Govindjee 1992) ΔV_t values of mock- and TeA-treated seedlings were derived (lower curves) [$V_t = (F_t - F_o)/(F_m - F_o)$]. Even though most parts of the ΔV_t curves of TeA- and mock-treated seedlings were identical, there was a minor, but significant increase of the 'J' point of TeA-treated seedlings relative to the control. (c,d) The effect of TeA on the JIP test parameters PI_{ABS} (c) and PHI(E_0) (d). Five-day-old light-grown seedlings were adapted to the dark for 30 min and then sprayed with 250 μM TeA or 0.1% DMSO. They were incubated afterwards in the dark for another 30 min, before they were re-exposed to moderate light at room temperature (90 μmol photons m^{-2} s^{-1}, 22 °C) for 3 h. Each curve/value shown in this Figure represents the average of three independent experiments with at least 20 repetitions of chlorophyll fluorescence measurements. Significant differences between different treatments ($P < 0.05$) have been verified by Duncan's multiple range test (SSR).

Five-day-old light-grown wild type and *ex1/ex2* seedlings were transferred to the dark for 30 min, sprayed with 250 μM TeA or a solvent lacking the toxin. At this TeA concentration the PSII electron transfer beyond Q_A is inhibited by 50% (Chen *et al*. 2007). Thirty minutes after spraying, plants were returned to continuous light for 3 h before fluorescence induction kinetics were recorded. The effects of TeA on relative chlorophyll fluorescence changes were normalized between F_o and F_m and plotted as V_t changes on a logarithmic scale [$V_t = (F_t - F_o)/(F_m - F_o)$]. The chlorophyll fluorescence rise kinetics of TeA- and mock-treated wild type and *ex1/ex2* seedlings looked very similar (Fig. 1a,b). From these individual OJIP curves ΔV_t values of mock- and TeA-treated seedlings were derived [$\Delta V_t = \Delta(F_t - F_o)/(F_m - F_o)$]. Even though

for most parts ΔV_t curves of TeA-treated seedlings looked similar to the control lines, they revealed a minor, but significant increase at the 'J' point, indicating that electron transfer on the acceptor side of PSII between Q_A and Q_B was slightly inhibited after seedlings had been exposed to 250 μM TeA (Fig. 1a,b). As shown by comparison of the ΔV_t curves of TeA-treated wild type and *ex1/ex2* seedlings, both types of seedlings showed a very similar response to TeA confirming our notion that the absence of EX1 and EX2 in *ex1/ex2* seedlings does not visibly modify the inhibitory effect of TeA on photosynthetic electron transport. The overall PSII photosynthetic activity and quantum yield for PSII electron transport beyond Q_A can be quantified by the JIP test parameters PI_{ABS} and $PHI(E_0)$, respectively (Strasser *et al*. 2004; Chen *et al*. 2007). TeA treatment caused a decrease of PI_{ABS} and $PHI(E_0)$ of *Arabidopsis* seedlings. However, there was no significant difference between wild type and *ex1/ex2* (Fig. 1c,d).

Analysis of $^1O_2$-responsive marker gene expression in TeA-treated *Arabidopsis* plants

Inhibition of the electron flow at the acceptor side of PSII in TeA-treated seedlings is expected to enhance formation of the triplet state of excited RC chlorophyll P680 and production of $^1O_2$. To confirm the predicted release of $^1O_2$, an attempt was made to measure this reactive oxygen species directly with the two probes dansyl-2,2,5,5-tetramethyl-2,5-dehydor-*1H*-pyrrole (Danepy) and singlet oxygen sensor green (SOSG) that had been previously used for $^1O_2$ detection (Hideg *et al*. 1998; Flors *et al*. 2006; Kim *et al*. 2012; Kim & Apel 2013a). However, the level of $^1O_2$ in seedlings treated with TeA for 3 h was too low to be detected by either probe. Thus, an alternate procedure was chosen to reveal the release of $^1O_2$ more indirectly by measuring expression changes of $^1O_2$-responsive marker genes (Baruah *et al*. 2009). In seedlings of the conditional *fluorescent* (*flu*) mutant of *A. thaliana* that upon a dark/light treatment generates $^1O_2$ in chloroplasts the earliest $^1O_2$-responsive marker gene expression changes had been reported 30 min after the onset of $^1O_2$ formation (op den Camp *et al*. 2003; Gadjev *et al*. 2006). However, more recent studies have shown that these nuclear gene expression changes in *flu* 30 min after the onset of $^1O_2$ production are preceded by a rapid EX-dependent loss of chloroplast integrity (Kim *et al*. 2012). Thus, many of the $^1O_2$-responsive genes reported earlier in the *flu* mutant are probably not under direct control of $^1O_2$-mediated and EX-dependent signalling, but instead are activated by other signalling pathways closely associated with cellular damage (Kim *et al*. 2012; Zhang *et al*. 2014). In the present study, this obstacle has been overcome by identifying $^1O_2$-responsive genes up-regulated already during the first 15 min of re-illumination of pre-darkened *flu* seedlings, while chloroplast integrity is still maintained. Two different approaches were used to identify suitable $^1O_2$-responsive marker genes (Fig. 2). First, RNA-seq-derived transcriptomes of light-grown *flu* and wild-type seedlings shifted to the dark for 8 h and re-exposed to light for 15, 30 and 120 min were determined (Fig. 2a). The number of genes up-regulated in *flu* relative to wild type rapidly increased from 404 after 15 min to 1.172 and 3.187 after 30 and 120 min of re-illumination, respectively (Kim & Apel 2013b). As chloroplast leakage in *flu* seedlings occurs already 30 min after the beginning of $^1O_2$ production, $^1O_2$-responsive marker genes were selected only from the 404 genes up-regulated 15 min after the dark/light shift for the subsequent analysis of gene expression changes in TeA-treated seedlings (Supporting Information Tables S1 & S3). In a second experiment, wild type and *ex1/ex2* seedlings initially grown under continuous light at 22 °C and 90 μmol photons m^{-2} s^{-1} were transferred to the dark for 30 min and then exposed to a combined lower temperature/higher light stress (12 °C and 270 μmol photons m^{-2} s^{-1}) for 24 h (Fig. 2b). In wild type, exposed to the lower

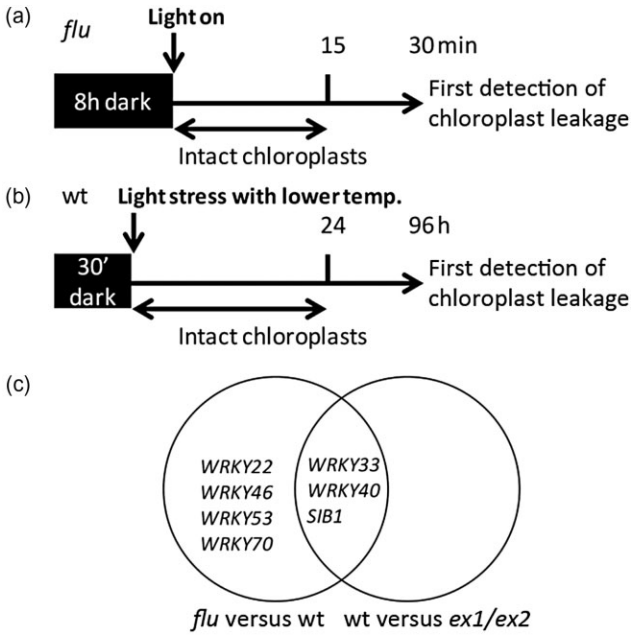

Figure 2. Schematic diagram showing the strategy used to identify early $^1O_2$- and EXECUTER (EX)-dependent marker genes. (a,b) Treatments of plants used for genome-wide search of $^1O_2$- and EX-dependent transcript changes in *flu* and wild type (wt) by RNA-seq. (a) Five-day-old *flu* and wt seedlings grown under continuous light were shifted to the dark for 8 h and re-exposed to light for various lengths of time. $^1O_2$-responsive marker genes were identified among genes up-regulated in *flu* but not in wt after 15 min of re-illumination, when chloroplasts in *flu* seedlings were still intact (Kim *et al*. 2012). (b) Five-day-old wt and *ex1/ex2* seedlings grown under continuous light at room temperature were shifted to the dark to allow the temperature to drop from 22 to 12 °C in the absence of light before the seedlings were exposed to the lower temperature/higher light stress as described previously (Kim *et al*. 2012). A loss of chloroplast integrity in these plants was first seen after 96 h of stress treatment (Kim *et al*. 2012). $^1O_2$-responsive marker genes were identified among the genes up-regulated in wt and suppressed in *ex1/ex2* during the first 24 h of stress treatment. (c) seven $^1O_2$-marker genes were selected from the early $^1O_2$-responsive genes of *flu* seedlings. Three of these genes were also found among the $^1O_2$- and EX-dependent genes of wt kept under lower temperature/higher light stress.

temperature/higher light stress, an EX-dependent loss of chloroplast integrity followed by cell death does not occur right after the onset of $^1O_2$ production as in the *flu* mutant, but only after an extended stress treatment of 96 h (Kim *et al*. 2012). Among the more than 2000 stress-responsive genes that were up-regulated in wild type after 24 h of stress treatment 69 were suppressed in *ex1/ex2* and only five of these 69 genes were also found among the 404 $^1O_2$-reponsive genes of the *flu* mutant that were up-regulated immediately before the loss of chloroplast integrity (Supporting Information Tables S2 & S3). Hence, most of the genes activated by $^1O_2$-mediated and EX-dependent signalling in wild type placed under stress that did not cause a rapid cell death response were not affected in *flu*, and vice versa only a minute fraction of $^1O_2$-responsive genes of the *flu* mutant was activated in wild type under lower temperature/higher light stress (Kim *et al*. 2012). To analyse whether TeA treatment of *Arabidopsis* seedlings would activate the $^1O_2$- and EX-dependent signalling pathway, seven marker genes were selected from the set of 404 $^1O_2$-responsive genes of the *flu* mutant that were activated prior to the onset of chloroplast leakage (Fig. 2c). Three of them, *SIB1*, *WRKY 33* and *WRKY40* were also activated in wild type under lower temperature/higher light stress (Figs 2c & 3a), whereas the expression of the other four genes, *WRKY22, WRKY46, WRKY53* and *WRKY70*, was only triggered in *flu* under conditions that led to chloroplast leakage and a subsequent cell death response (Figs 2c & 3b). In addition to these seven genes, *AAA-ATPase*, which had also been previously used as a $^1O_2$-responsive marker gene (Baruah *et al*. 2009), was included in this study.

Five-day-old seedlings grown under continuous light were transferred to the dark for 30 min before they were sprayed with 250 μM TeA or a solvent lacking this toxin. After treatment, plants were kept in the dark for 30 min before they were returned for 3 and 6 h to light or they were left in the dark for up to 6 h (Fig. 4a). In mock-treated seedlings, transcripts of selected $^1O_2$-responsive marker genes were not detected except for trace amounts of transcripts of *WRKY22* and *WRKY70* after 3 and 6 h of illumination (Fig. 4b,c). Thus, physical stress because of the handling of seedlings during spraying hardly affected the expression of the marker genes (Fig. 4b,c). In TeA–treated seedlings kept in the dark transcripts of *AAA-ATPase*, *SIB1*, *WRKY33* and *WRKY40* accumulated transiently at 3 h after spraying, but were no longer detectable at 6 h after the treatment (Fig. 4b). In TeA-treated plants kept in the light, these transcripts also showed their maximum after 3 h of TeA treatment, but reached much higher levels than in seedlings kept in the dark, and declined thereafter (Fig. 4b). On the other hand, transcripts of *WRKY22*, *WRKY46*, *WRKY53* and *WRKY70* were hardly detectable in TeA-treated seedlings kept in the dark, but in illuminated seedlings were strongly up-regulated and reached similar high levels after 3 h of TeA treatment as the other $^1O_2$-responsive marker genes (Fig. 4c). Collectively, these results show that treatment with 250 μM TeA induced a rapid and transient transcript accumulation that in the case of *WRKY22*, *WRKY46*, *WRKY53* and *WRKY70* seemed to be light-dependent (Fig. 4c) or – in case of the other marker genes – was greatly accelerated under light conditions (Fig. 4b). These findings are in line with the notion that TeA induces expression changes of $^1O_2$-responsive genes primarily because of its inhibition of light-driven photosynthetic electron transport at the acceptor side of PSII that evokes an enhanced generation of $^1O_2$.

The possible involvement of $^1O_2$-mediated and EX-dependent signalling during TeA-induced gene expression changes has been analysed by comparing the effect of TeA treatment on the expression of the $^1O_2$-responsive marker genes in wild type and *ex1/ex2* seedlings. Expression of *WRKY22*, *WRKY46*, *WRKY53* and *WRKY70*, but also *WRKY40*, is strongly suppressed in TeA-treated *ex1/ex2* seedlings suffering from a block in $^1O_2$-mediated and EX-dependent signalling (Fig. 5a,b,d). Transcripts of the $^1O_2$-responsive marker genes *AAA-ATPase*, *SIB1* and *WRKY33* accumulated also in TeA-treated *ex1/ex2* seedlings, but levels of these transcripts were not as high as in TeA-treated wild-type seedlings (Fig. 5b). These conclusions were confirmed by plotting mean values of transcript levels derived from three independent experiments against the time of TeA treatment (Fig. 5c,e).

In the *flu* mutant up-regulation of these $^1O_2$-responsive marker genes is followed by an almost instantaneous loss of chloroplast integrity and a subsequent collapse of the central vacuole, whereas in wild type placed under lower temperature/higher light stress $^1O_2$-mediated and EX-dependent signalling does not induce such an immediate change in chloroplast integrity (Kim *et al*. 2012). Chloroplast integrity in *flu* and TeA-treated wild-type seedlings was assessed under the confocal microscope by monitoring the distribution of the GFP in transgenic plants that express a reporter protein consisting of the GFP and the small subunit of the ribulose-1,5-bisphosphate carboxylase (SSU) that directs the GFP to the chloroplast compartment (Kim & Apel 2004). In *flu* seedlings grown under continuous light, the fusion protein was confined to the chloroplast (Fig. 6a). Following a dark-to-light shift that promotes $^1O_2$ production in chloroplasts of *flu* seedlings (op den Camp *et al*. 2003), chloroplast integrity was rapidly lost as indicated by the leakage of the GFP into the surrounding cytoplasm (Fig. 6a). However, in TeA-treated wild-type seedlings, chloroplasts remained intact throughout the rapid increase and subsequent decline of transcript levels of the eight selected $^1O_2$-responsive genes, as shown by the intracellular distribution of GFP fluorescence (Fig. 6b). Collectively, these results demonstrate that activation of the $^1O_2$- and EX-dependent signalling pathway leads to a rapid up-regulation of the *WRKY 22*, *46*, *53* and *70* genes in *flu* following a dark-to-light shift, but not in wild type exposed to the lower temperature/higher light stress (Fig. 3b). However, similar to *flu*, in wild type treated with TeA, these genes were rapidly up-regulated, but unlike *flu*, TeA-treated seedlings did not show the $^1O_2$-mediated cell death response. These results emphasize the important role of the physiological context that leads to an enhanced generation of $^1O_2$ in modifying the plant's response to $^1O_2$-mediated signalling.

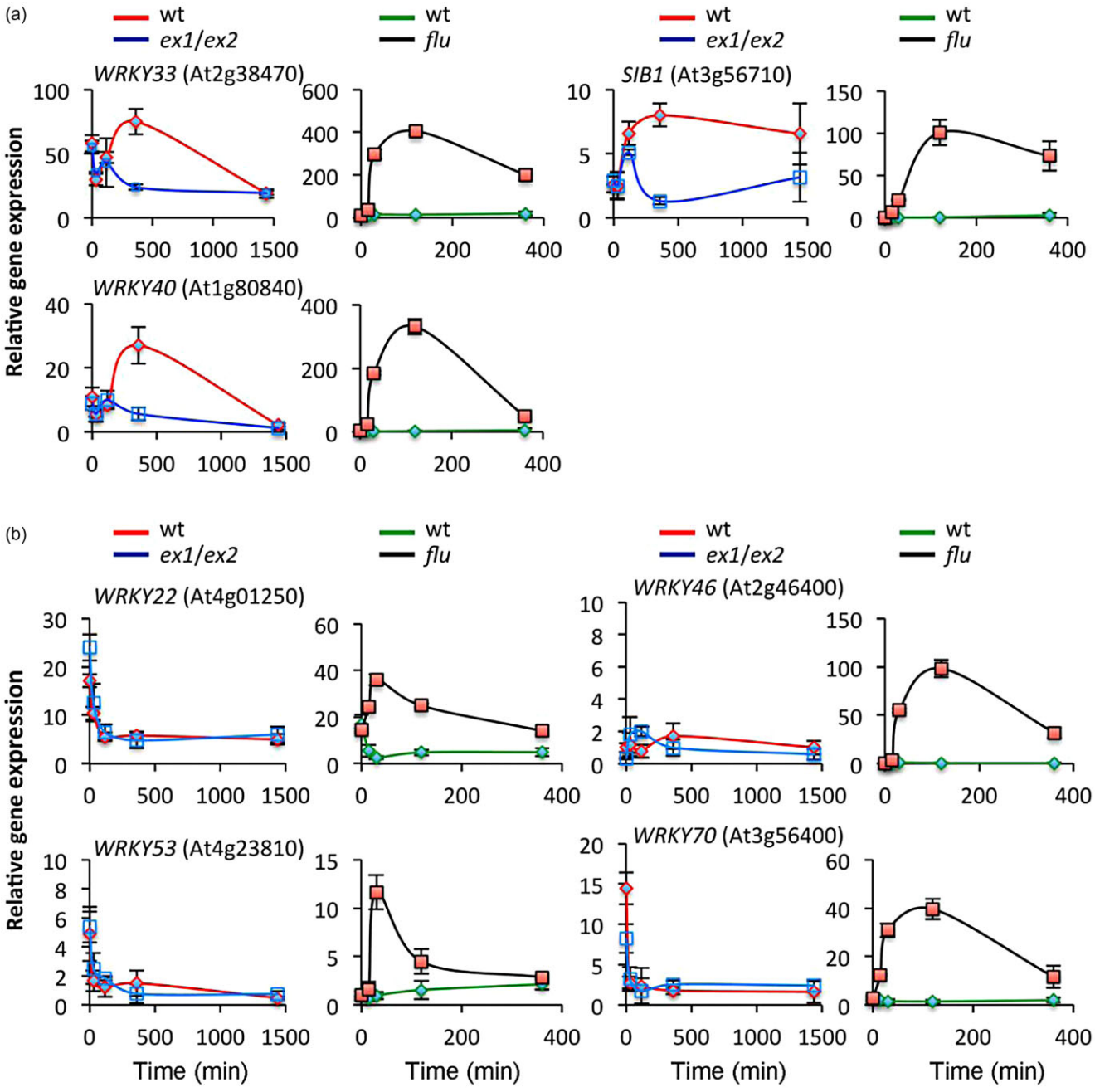

Figure 3. $^1O_2$-mediated and EXECUTER (EX)-dependent expression changes of the selected marker genes shown in Fig. 2c. (a) Activation of *WRKY33*, *SIB1* and *WRKY40* gene expression in wild type (wt) exposed to a combined lower temperature/higher light stress as compared with *ex1/ex2* and in *flu* seedlings following an 8 h dark/light shift as compared with wt. (b) Activation of *WRKY22*, *WRKY46*, *WRKY53* and *WRKY70* gene expression in the *flu* seedlings following an 8h dark/light shift as compared with wt. These genes were not up-regulated in wt or *ex1/ex2* seedlings exposed to the combined lower temperature/higher light stress. The standard deviation of individual gene expression values was obtained using three independent biological samples.

$^1O_2$-mediated and EX-dependent responses of TeA-treated seedlings

Three days after spraying with 250 μM TeA, wild-type seedlings did not show any of the $^1O_2$-mediated cell death responses of the *flu* mutant (op den Camp *et al*. 2003; Wagner *et al*. 2004; Lee *et al*. 2007). To enhance the stress level, seedlings were sprayed with increasing concentrations of TeA ranging from 250 μM to 2 mM and were kept in the light after the treatment for longer periods of time. Three days after the treatments, the chlorophyll fluorescence induction kinetics and also the ΔV_t curves derived from the normalized fluorescence changes were greatly affected and revealed major impairments of the photosynthetic electron transport in

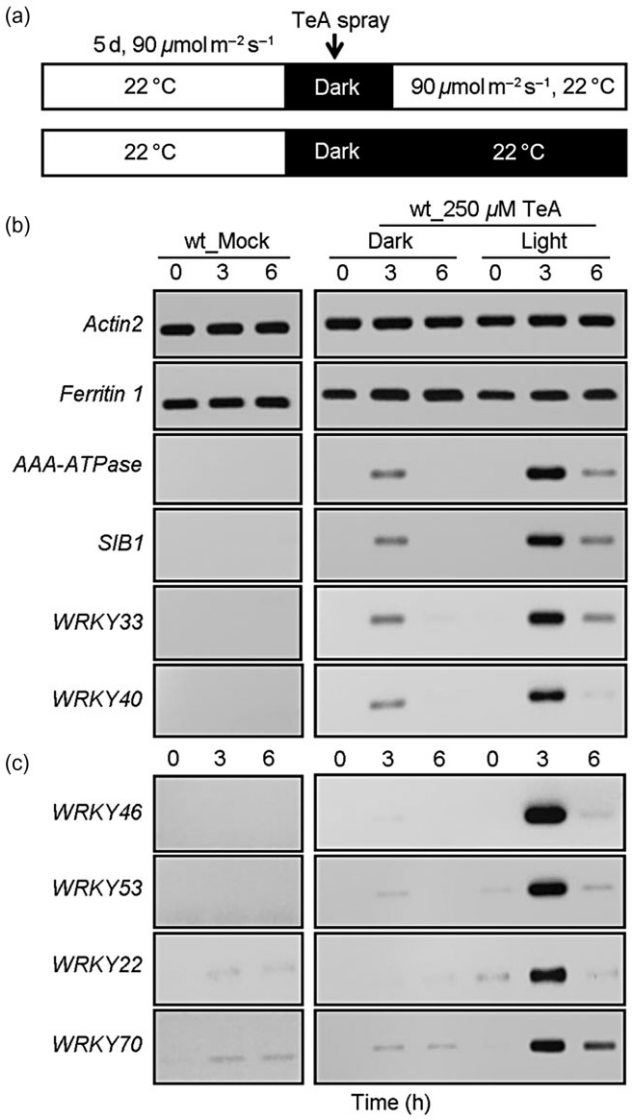

Figure 4. Light-dependent changes of $^1O_2$-responsive gene expression in tenuazonic acid (TeA)-treated seedlings of *Arabidopsis thaliana*. (a) Seedlings grown for 5 d under continuous light (90 μmol photons m^{-2} s^{-1}) at 22 °C were transferred to the dark for 1 h, sprayed with 250 μM TeA or 0.1% dimethyl sulphoxide (DMSO), incubated for 30 min in the dark and then re-exposed for 3 or 6 h to the moderate light or kept for 3 and 6h in the dark. (b) TeA-induced transcript changes of $^1O_2$-responsive marker genes that are up-regulated both in the *flu* mutant of *Arabidopsis* within 15 min following the release of $^1O_2$ and in wild-type seedlings exposed to a combined lower temperature/higher light stress for up to 24 h. As shown previously the combined lower temperature/higher light stress treatment activates $^1O_2$-mediated and EXECUTER (EX)-dependent signalling in wild type (wt; Kim *et al*. 2012). Seedlings were frozen under liquid nitrogen at different time points as shown in (a). Total RNA was extracted and changes in gene expression visualized by RT-PCR using gene-specific primers. Controls include RNA samples from mock-treated illuminated seedlings and TeA-treated seedlings kept in the dark, and transcript measurements of *AAA-ATPase* ($^1O_2$-responsive marker gene), *Ferritin1* (H_2O_2-responsive marker gene) and *Actin2* (loading control). (c) TeA-induced transcript changes of $^1O_2$-responsive marker genes that are up-regulated in the *flu* mutant within 15 min following the release of $^1O_2$, but not in wt seedlings exposed to the lower temperature/higher light stress. The $^1O_2$-mediated gene expression changes in the *flu* mutant occur prior to the rapid loss of chloroplast integrity, whereas in wt exposed to the lower temperature/higher light stress no detectable cellular damage occurs throughout 24 h of stress treatment (Kim *et al*. 2012). The experimental conditions were the same as in (b).

TeA-treated wild-type seedlings (Fig. 7a). In *ex1/ex2* seedlings treated in the same way as wild type with increasing TeA concentrations, these impairments were far less severe (Fig. 7b). Impairment of PSII photosynthetic activity in TeA-treated seedlings was also revealed by measuring the JIP test parameters PI_{ABS} and $PHI(E_0)$ (Fig. 7c,d). Long-term effects of TeA on PSII again were less obvious in *ex1/ex2* than in wild-type seedlings (Fig. 7c,d). Three days after spraying with 1 or 2 mM TeA, wild-type seedlings developed lesions that in *ex1/ex2* seedlings were reduced, but not completely suppressed (Fig. 8). These results demonstrate that following treatment with TeA $^1O_2$-mediated and EX-dependent signalling is activated and may trigger lesion formation. However, lesion formation in TeA-treated seedlings cannot be exclusively attributed to $^1O_2$-mediated and EX-dependent signalling. This conclusion is also in line with the impairment of overall PSII photosynthetic activity (PI_{ABS}) and quantum yield for PSII electron transport beyond Q_A [$PHI(E_0)$] in wild type after 3 d of TeA treatment that is less severe, but not completely suppressed in *ex1/ex2* seedlings (Fig. 7c,d).

DISCUSSION

A major finding of the present study is that TeA, a toxin found in several phytopathogenic fungi, such as *A. alternata*, activates the $^1O_2$- and EX-dependent signalling pathway in chloroplasts of *A. thaliana*. In previous work, TeA had been reported to exhibit a broad range of antiviral, antitumour and antimicrobial activities (Yuki *et al*. 1967), and to inhibit protein synthesis in eukaryotic cells (Friedman *et al*. 1975). Furthermore, in plants, it was also shown to block seed germination (Tylkowska *et al*. 2003) and to reduce the growth of seedlings (Marfori *et al*. 2003). However, these studies did not reveal a primary target of the toxin during the interaction of the pathogen with its host plants. More recently, TeA has been shown to impede the photosynthetic electron transport at the acceptor side of PSII by blocking the Q_B-binding site on the D1 protein of PSII RC (Chen *et al*. 2007). The results of the present study are in line with these latter findings and offer clues as to how TeA by targeting this step of the photosynthetic electron transport may promote a cell death response.

A block of the Q_B-binding site by TeA does not only induce the accumulation of reduced Q_A, but is also expected to interfere with the transfer of electrons from the reduced excited P680 chlorophyll of PSII RC to the primary electron acceptor of PSII, phaeophytin (Vass & Cser 2009). This impairment favours formation of the triplet state of the chlorophyll and the transfer of its excitation energy onto ground state triplet oxygen giving rise to $^1O_2$ (Vass & Cser 2009). In

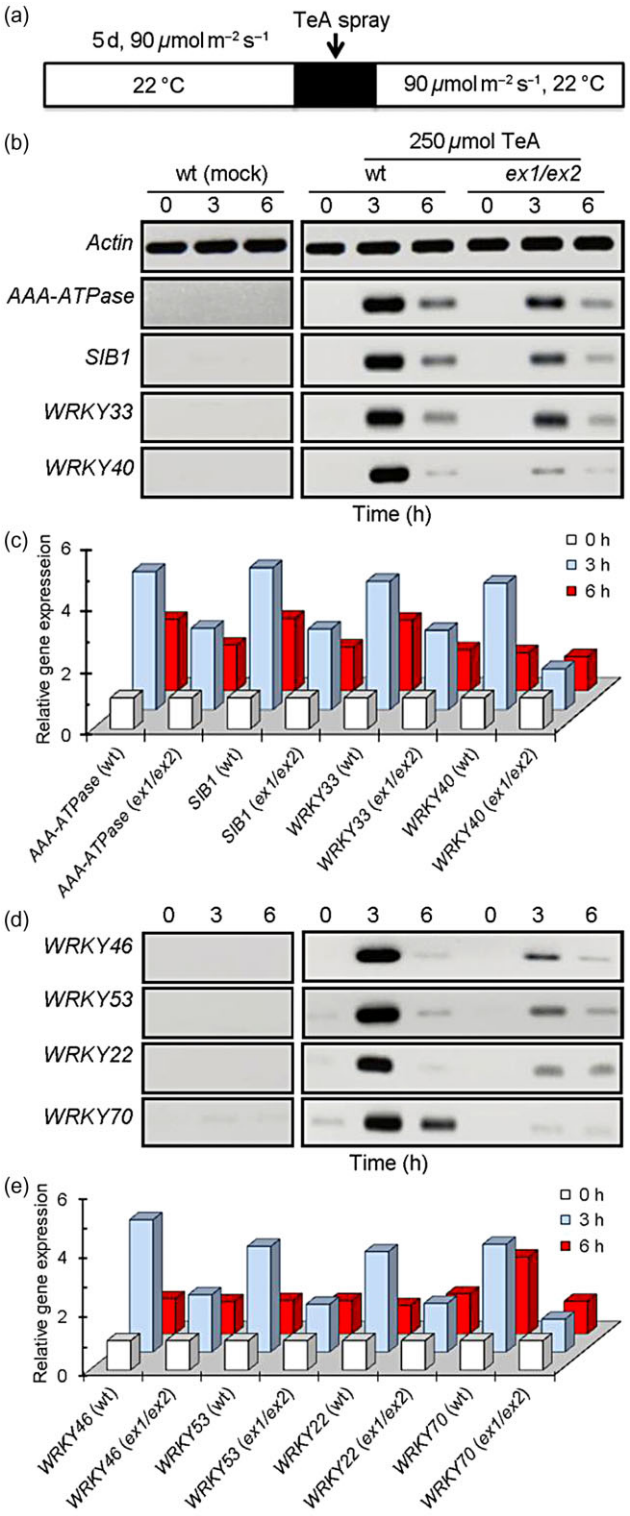

Figure 5. Tenuazonic acid (TeA)-induced changes of $^1O_2$-responsive marker gene expression in wild type and *ex1/ex2* seedlings of *Arabidopsis thaliana*. (a) Wild type (wt) and *ex1/ex2* seedlings of *Arabidopsis* were treated with TeA as shown in Fig. 4a except that following the treatment and a 30 min dark incubation all seedlings were re-exposed to moderate light for 3 or 6 h. (b) TeA-induced transcript changes of $^1O_2$-responsive marker genes shown in Fig. 4b. (d) TeA-induced transcript changes of $^1O_2$-responsive marker genes shown in Fig. 4c. Controls include RNA samples from mock-treated seedlings and transcript levels of the *Actin2* gene (loading control). (c,e) Quantification of TeA-induced transcript changes of $^1O_2$-responsive marker genes shown in (b) and (d), respectively. Three biological replicas were analysed by RT-PCR and the mean values are shown. Data are normalized to the values measured in the mock-treated wild type at time '0'. Standard deviations of these values are given in Supporting Information Table S7.

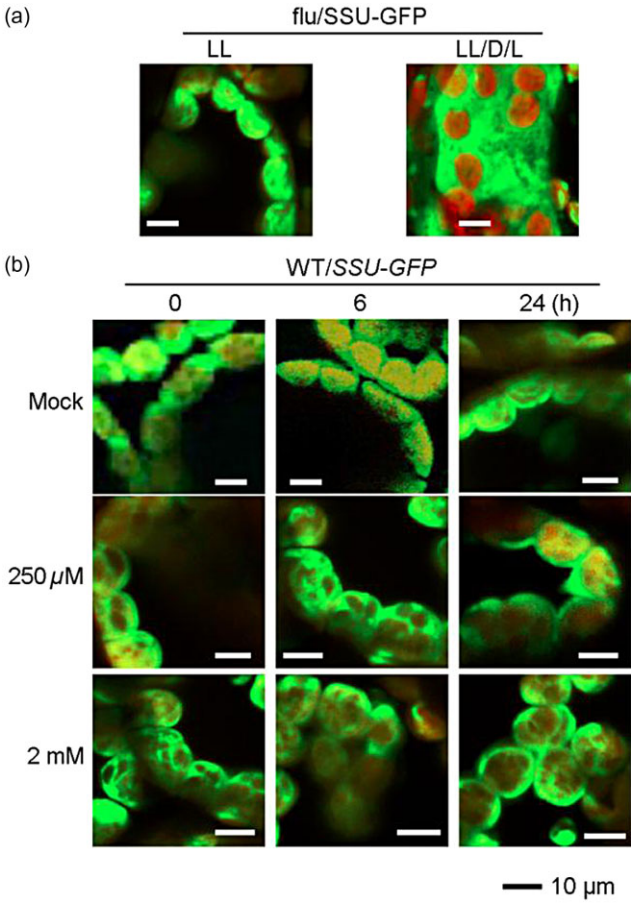

Figure 6. The assessment of chloroplast integrity by confocal microscopy in seedlings expressing the SSU–GFP fusion protein. (a) Five-day-old *flu* seedlings were either grown under continuous light (LL) or shifted to the dark for 8 h and re-exposed to light for 1 h (LL/D/L). (b) Five-day-old wild-type seedlings initially grown under continuous light were treated with different concentrations of 250 μM or 2 mM tenuazonic acid (TeA) as described in Fig. 4a and the chloroplast integrity was monitored after 6 and 24 h of TeA treatment. Note that *flu* seedling show a dramatic chloroplast leakage already after 1 h of re-illumination, whereas in TeA-treated wild-type seedlings chloroplasts remained intact throughout 24 h of TeA treatment. Bar size: 10 μm.

chloroplasts, $^1O_2$ may act in at least two different ways. Under severe stress conditions $^1O_2$ is more likely to interact directly with proteins, nucleic acids, lipids and carotenoids, causing oxidative damage (Triantaphylidès & Havaux 2009). Under less severe stress conditions, $^1O_2$ may activate in chloroplasts also the EX-dependent signalling pathway (op den Camp

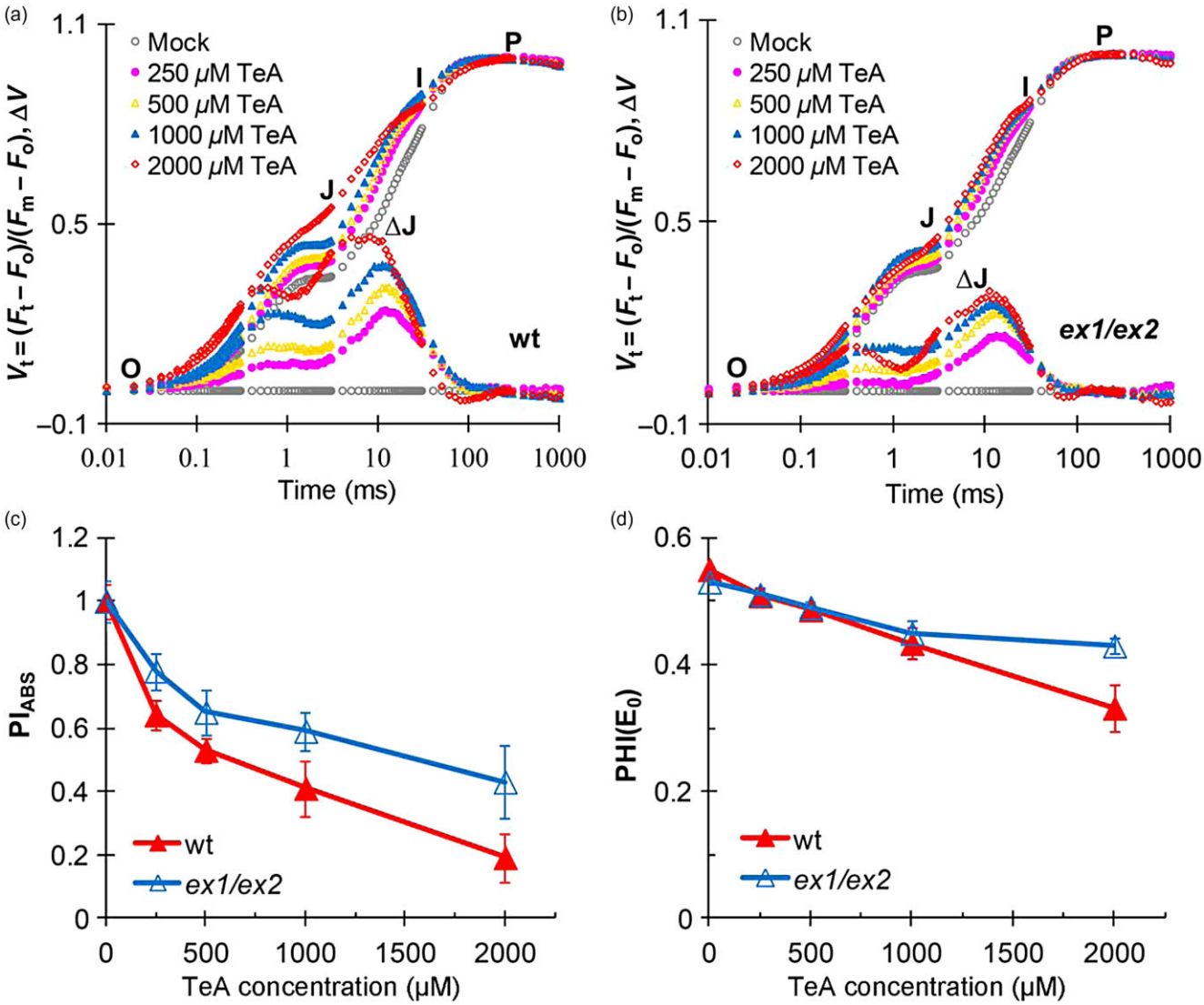

Figure 7. Chlorophyll fluorescence induction kinetics of (a) wild type (wt) and (b) *ex1/ex2* seedlings of *Arabidopsis thaliana* treated with tenuazonic acid (TeA). The experimental conditions are the same as shown in Fig. 1 except that seedlings were treated with different TeA concentrations for 3 d before the relative fluorescence rise was determined. (c,d) the effect of different TeA concentrations on the JIP parameters PI_{ABS} (c) and $PHI(E_0)$ (d) determined in seedlings 3 d after the beginning of the TeA treatment. Each curve/value shown in this Figure represents the average of three independent experiments with at least 20 repetitions of chlorophyll fluorescence measurements. Significant differences between different treatments ($P < 0.05$) have been verified by Duncan's multiple range test.

et al. 2003; Wagner *et al*. 2004; Lee *et al*. 2007). Initially, $^1O_2$-mediated and EX-dependent signalling had been studied in the conditional *flu* mutant of *Arabidopsis* that upon a dark-to-light shift generates $^1O_2$ within chloroplasts. It was thought that $^1O_2$-mediated gene expression changes are under direct control of the $^1O_2$- and EX-dependent signalling pathway (Kim *et al*. 2008). However, subsequent studies of the *flu* mutant revealed a more complex picture. The onset of $^1O_2$ formation is rapidly followed by a loss of chloroplast integrity and the rupture of the central vacuole that precede the expression changes of $^1O_2$-responsive nuclear genes reported earlier (Kim *et al*. 2012). Hence, many of these genes are likely to be only indirectly affected by $^1O_2$-mediated signalling and seem to be under direct control of other signalling pathways that are activated during the loss of cellular integrity (Baruah *et al*. 2009). For the present study only $^1O_2$-responsive genes have been considered that were up-regulated prior to the $^1O_2$-mediated loss of chloroplast integrity. Most of these early $^1O_2$-responsive nuclear genes of *flu* seem to be associated with the initiation of chloroplast leakage and the subsequent collapse of the cell. This conclusion is based primarily on our finding that the expression of almost all of these genes is not affected in wild-type seedlings exposed to a mild combined lower temperature/higher light stress that evokes $^1O_2$ formation. Superficially, $^1O_2$-responsive genes of wild type seem to be associated with similar physiological processes as the $^1O_2$-responsive genes in the *flu* mutant (Supporting Information Figure S1). However, a

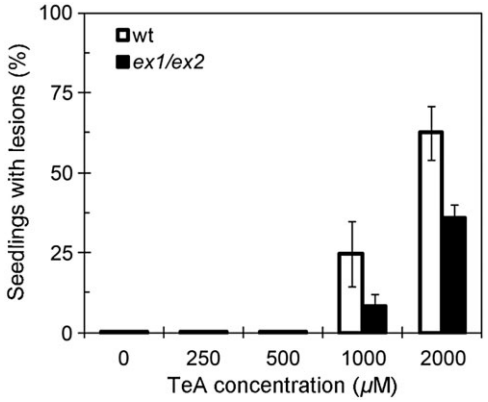

Figure 8. Tenuazonic acid (TeA)-induced bleaching of wild type (wt) and *ex1/ex2* seedlings of *Arabidopsis thaliana*. Five-day-old seedlings were sprayed with different concentrations of TeA, as described in Fig. 5a, and re-exposed to moderate light for 3 d before the percentage of bleached seedlings was determined. Each value represents the average of three independent experiments using a minimum of 100 seedlings for each sample.

closer look revealed significant differences between different sets of genes of the *flu* mutant and wild type. For instance, activated genes associated with 'electron transport or energy pathway' in wild type encode proteins involved in light harvesting, whereas in *flu*, genes belonging to this category can be linked to components of the photosynthetic and respiratory electron transport chains (Supporting Information Tables S5 & S6). Similarly, genes that form part of the functional category 'transcription and DNA-dependent' in *flu* encode various WRKY and zinc finger transcription factors and ethylene response DNA-binding factors, whereas in wild type, exposed to mild light stress, $^1O_2$-responsive genes of this category seem to be primarily associated with ABA signalling (Supporting Information Tables S5 & S6). There are at least two possible reasons why $^1O_2$-responsive genes in wild type and *flu* differ. First, in wild type exposed to mild light stress, $^1O_2$-mediated signalling activates gene expression changes without inducing a concomitant loss of chloroplast integrity and a cell death response (Kim *et al*. 2012). Hence, $^1O_2$-responsive genes that in *flu* may participate in triggering an immediate cell death response or are activated by signalling pathways associated with cellular damage are probably not affected in these wild-type plants. Secondly, $^1O_2$-mediated signalling in *flu* operates as a default pathway independently of other signalling pathways, whereas in wild type exposed to the lower temperature/higher light stress $^1O_2$-mediated signalling is not activated alone as in *flu*, but together with other signalling pathways that interact with $^1O_2$- and EX-dependent signalling and modify its specificity (Laloi *et al*. 2007; Simkova *et al*. 2012). Such modifications are likely to impact the expression of $^1O_2$-responsive genes.

To analyse the effect of TeA on $^1O_2$-responsive gene expression in *Arabidopsis* seedlings, two sets of genes were selected that were either up-regulated both in wild type in response to the lower temperature/higher light stress and in *flu* following a dark-to-light shift or exclusively in the *flu* mutant prior to the onset of chloroplast leakage. The former group consisted of *SIB1*, *WRKY33* and *WRKY40*, the latter of *WRKY22*, *WRKY46*, *WRKY53* and *WRKY70*. Expression changes of the first group of genes are not exclusively dependent on the release of $^1O_2$ in chloroplasts and are controlled also by other, as yet unknown light-independent signalling events that are triggered by TeA, whereas TeA-induced expression changes of the second group of $^1O_2$-responsive marker genes seem to be exclusively controlled by the $^1O_2$- and EX-dependent signalling pathway. In the *flu* mutant, expression of these genes precedes a rapid loss of chloroplast integrity, the first step of a $^1O_2$-mediated programmed cell death response. However, in TeA-treated seedlings, chloroplasts remained intact and no immediate cell death response could be observed that might be linked to the EX-dependent up-regulation of these early $^1O_2$-responsive marker genes.

In wild type exposed to the combined lower temperature/higher light stress, $^1O_2$-mediated and EX-dependent signalling induces only after an extended period of stress treatment the formation of microlesions (Kim *et al*. 2012). These microlesions resemble cell death responses closely associated with an enhanced resistance against biotrophic pathogens (Alvarez *et al*. 1998). Such locally restricted cell death responses dubbed 'hypersensitive response' (HR) are thought to confine the spread of biotrophic pathogens by abolishing the nutrient supply and limiting the growth of the pathogen (Mengiste 2012). Necrotrophic fungal pathogens have been shown to exploit such HR-cell death responses to colonize the plant (Lorang *et al*. 2012). Also in *Arabidopsis* seedlings treated with TeA for an extended period of 3 d, a cell death response was induced. It is tempting to speculate that TeA, by targeting the acceptor side of PSII in chloroplasts of a host plant, activates a $^1O_2$- and EX-dependent programmed cell death response that may help the fungus to colonize the host. However, such a cell death response was seen only at a higher concentration of TeA than needed to activate the $^1O_2$- and EX-dependent signalling pathway and it was not completely suppressed in TeA-treated *ex1/ex2* seedlings. Hence, in *Arabidopsis* an EX-dependent programmed cell death response triggered by $^1O_2$ at higher TeA concentrations is superimposed by other signalling events. As TeA is only one of numerous bioactive molecules that are released by the fungus during its interaction with a host, lesion formation and cell death responses of the plant are probably not exclusively triggered by TeA alone, but by a mixture of bioactive components that besides low molecular weight metabolites such as TeA include also for example proteins and bioactive saccharides (Mengiste 2012; Lou *et al*. 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Functional categories of genes up-regulated only in *flu* (*flu*-specific) upon a dark-to-light transition or wild type (wt-specific) exposed to the combined lower temperature/higher light stress. The enrichment of up-regulated genes in different functional categories of the biological processes has been obtained via http://www.arabidopsis.org/tools/bulk/go/index.jsp.

Table S1. List of genes that are up-regulated in *flu* mutant at levels at least twofold higher than wild-type seedlings during 15 min of re-illumination following 8 h dark.

Table S2. List of genes that are specifically up-regulated in wild type at levels at least twofold higher than in *ex1/ex2* seedlings under the combined lower temperature/higher light stress condition.

*Maximum fold change among all time points upon stress (30, 120, 360 and 1440 min) has been selected.

Table S3. List of genes that are up-regulated in both *flu* during 15 min of re-illumination following 8h dark and wild type under the combined lower temperature/higher lights stress condition (at least a twofold increase).

Table S4. List of genes and primer sequences used for the gene expression analysis by semi-quantitative PCR.

Table S5. Functional categories of genes up-regulated in *flu* compared with wild type during 15 min of re-illumination following 8 h dark.

Table S6. Functional categories of genes up-regualted in wild type compared with *ex1/ex2* under the combined low temperature/higher light stress.

Table S7. Standard deviation of transcriptional levels shown in Fig. 5c,e.