Plant-to-plant reactive oxygen signal transmission via a *Cuscuta* bridge

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Abstract

Cuscuta, commonly known as dodder, is a parasitic plant that thrives by attaching itself to the stems of other plants. It is found across the globe and is notorious for obstructing crop growth as a weed. Over the past decade, *Cuscuta* has been used to gain insights into plant-plant interactions and molecular trafficking. Here, we report that two plants connected via a *Cuscuta* bridge can exchange rapid systemic calcium, electric, and reactive oxygen species signals. These findings suggest that plant interactions with *Cuscuta* may have beneficial effects to plants, enabling them to rapidly communicate with each other.

1. Introduction

Reactive Oxygen Species (ROS) are important signaling and/or byproduct molecules involved in various processes in plants (Mittler *et al.*, 2022). ROS encompass both free radicals and non-radical species containing oxygen, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). They play a crucial role in regulating numerous processes in plants, including growth, development, plant-pathogen interactions, and stress responses (Mittler *et al.*, 2022). However, inadequate control of ROS levels can result in cellular damage and oxidative stress, leading to cell death and tissue impairment. To maintain ROS homeostasis, plants evolved various mechanisms for regulating ROS production and scavenging (Mittler *et al.*, 2022).

The term 'ROS wave' refers to the temporal and spatial auto propagation of ROS production in plant tissues (Miller *et al.*, 2009; Mittler *et al.*, 2022). It is a phenomenon in which ROS production is induced in certain cells in response to a stimulus, such as biotic or abiotic stress, and spreads through the entire plant in a wave-like manner, triggering various physiological responses. During this process, each cell in the path of the ROS wave starts generating ROS, stimulating the cells around it and driving the wave forward. ROS waves have been observed in several major model plants, including *Arabidopsis*, maize, and tomato (Fichman *et al.*, 2019), but not yet in parasitic plants. The wave-like propagation of ROS has been shown to be crucial for coordinating plant responses to stress and initiating defense mechanisms against pathogens or environmental stresses, termed 'systemic acquired resistance' or 'systemic acquired acclimation', respectively (Mittler *et al.*, 2022). Intriguingly, the ROS waves can be visualized in whole intact plants by utilizing fluorescent dyes or genetically engineered biosensors designed to detect alterations in ROS concentrations (Miller *et al.*, 2009; Mittler *et al.*, 2022). By monitoring the progression of the ROS wave, its kinetics and dynamics can be unveiled, providing valuable insights into the intricate nature of ROS communication.

Parasitic plants "steal" water and nutrients from host plants after forming physical connections with them called haustorium (Hibberd & Dieter Jeschke, 2001). Over the past decade, investigations have used the stem parasitic plant *Cuscuta* (also known as dodder) to understand the transfer of mRNAs, small RNAs, DNA, and proteins between the host and *Cuscuta*, facilitated through the haustorium connection (Jhu & Sinha, 2022). Mobility of mRNAs

and proteins between two different plants connected by *Cuscuta* haustoria has also been shown (Liu *et al.*, 2020). In addition to macromolecules, the transfer of systemic herbivory signals has been observed from invaded plants to uninvaded plants when multiple hosts were connected by *Cuscuta* bridges (Hettenhausen *et al.*, 2017; Zhuang *et al.*, 2018).

In the current study, we investigated the transmission of stress-activated ROS, calcium, and membrane potential waves from 'donor' to 'receiver' plants by means of a *Cuscuta* 'bridge'. Using a live imaging system, we visualized and quantified changes in ROS, intracellular calcium levels, and membrane potential in plant-*Cuscuta*-plant connections in response to stress. In addition, qRT-PCRs showed changes in the steady-state level of transcripts associated with ROS in donor and acceptor plants, as well as in the *Cuscuta* bridge. These results provide direct evidence for the transmission of rapid systemic stress signals from a stressed tissue in one plant to another plant via a *Cuscuta* bridge.

2. Results and Discussions

Plant-Cuscuta-plant ROS signaling

In the interaction between host and *Cuscuta*, ROS were found to play vital roles in the host's defense mechanisms (Hegenauer *et al.*, 2016; Slaby *et al.*, 2021). *Cuscuta* is thought to induce ROS generation in the host plant, triggering a defense response that involves secondary metabolite production, reinforcement of the cell wall, and programmed cell death in infected tissues (Johnsen *et al.*, 2015; Hegenauer *et al.*, 2016). Moreover, ROS production during *Cuscuta* parasitism has been shown to influence behavior of the parasite and the ethylene signaling pathway in the host plant, thereby facilitating the penetration of the *Cuscuta* haustorium into the host tissues (Hegenauer *et al.*, 2016). ROS have also been shown to play a role in the regulation of gene expression in *Cuscuta*. Transcriptome analysis revealed differential expression of genes related to ROS metabolism in *Cuscuta* during the attachment and invasion of the host plant (Pan *et al.*, 2022). Although ROS has been studied as an integral part of the host-parasite interaction, the concept of the ROS wave remains unclear in these interactions.

To observe the propagation the ROS wave in response to wounding stress, we produced a 'donor' *Arabidopsis*-'bridge' *Cuscuta*-'receiver' *Arabidopsis* inter-plant (plant-*Cuscuta*-plant) system (Figure 1a). Both host and *Arabidopsis* plants were connected to *Cuscuta* through fully developed haustoria (Figure 1b, black arrow). This was done to prevent ROS signals involved in

79 the initial *Cuscuta*-plant interactions from interfering with the ROS wave detection assay. Using
80 a live imaging system (IVIS Lumina S5), the ROS wave was detected after wounding of a single
81 rosette leaf of the donor *Arabidopsis* (Figure 1a, yellow lightning bolt). Interestingly, the
82 accumulation of ROS was not limited to the local leaf of the donor plant but rather spread to and
83 throughout the donor systemic leaves, *Cuscuta* bridge, and to the entire receiver plant (Figure
84 1c). This finding suggested that the wounding of a single leaf of the donor plant triggered a
85 systemic ROS wave that traveled through the *Cuscuta* bridge and enhanced ROS accumulation
86 in the receiver plant. Importantly, all stressed tissues exhibited significantly higher ROS contents
87 (1.3-to-1.6-fold increase, in average) compared to their respective control conditions (Figure 1d).

88 To prevent potential interference from the ROS signaling processes associated with the
89 continuous penetration of *Cuscuta* haustoria into host stems (which is a type of wounding stress),
90 high light stress was implemented as a trigger of the ROS wave (Figure 2). Applying high light
91 stress to a single rosette leaf of a donor *Arabidopsis* plant led to ROS accumulation within 30
92 min in the entire plant-*Cuscuta*-plant chain including the donor, *Cuscuta*, and receiver plants
93 (Figure 2a-2b and Movie S1). As the ROS wave is frequently accompanied by the propagation of
94 systemic calcium and electric waves (Fichman & Mittler, 2021), we repeated the same
95 experiments and used the real-time imaging system and the plant-*Cuscuta*-plant chain (Figure
96 1a) with dye indicators for calcium and membrane potential changes. Similar to the ROS wave
97 observation, we were able to image and measure the propagation of a calcium wave (Figure 2c-
98 2d and Movie S2), and an electric wave characterized by membrane depolarization (Figure 2e-2f
99 and Movie S3), across the entire plant-*Cuscuta*-plant system. Taken together, our findings reveal
100 that ROS, calcium, and membrane potential waves, activated by a high light stress applied to one
101 tissue (leaf) of the donor plant, are transmitted to a receiver plant via the *Cuscuta* bridge. These
102 findings indicate that systemic stress signals (ROS, calcium, and electric waves) can be
103 transferred within minutes aboveground between different plants through a parasitic plant such
104 as *Cuscuta*.

105 **The plant-*Cuscuta*-plant ROS wave signal is accompanied by the activation of**
106 **transcriptional responses in the *Cuscuta* bridge and the receiver plant**

107 To investigate whether the ROS wave is accompanied by the triggering of molecular
108 responses upon transmission to other plants, we performed qRT-PCRs using total RNA extracted

from our experimental setup (Figure 1a). Tissues were harvested before and after high light stress, and transcript levels were compared relative to control condition. The qRT-PCRs results revealed an increase in the expression of *AtAPX2*, *AtMYB30, AtZAT10, AtZAT12,* and *AtZHD5* transcripts in both donor and receiver *Arabidopsis* plants under the high light stress (Figure 2g). These transcripts are known to accumulate in local and systemic tissues of *Arabidopsis* in response to a 2 min local light stress (Fichman *et al.*, 2022). The enhanced transcript expression in receiver plants suggest that the propagation of systemic signals through the *Cuscuta* bridge stimulates a molecular response in them. Furthermore, we investigated changes in the steady-state levels of the stress-associated *Cuscuta* transcripts, *CcCSD1*, *CcNDPK2*, and *CcGLR2.7,* to understand the potential influence of systemic waves on *Cuscuta*. Our qRT-PCR result revealed significant changes in the expression of these transcripts (Figure 2h), indicating that the transmitted systemic signals from the host plant could also trigger transcriptional responses in *Cuscuta*.

3. Summary

In a recent study, we demonstrated that under humid conditions plants growing in a community can transfer ROS wave signals between each other, given that their leaves are physically touching each other (Szechyńska-Hebda *et al.*, 2022). Here we extend our findings and report that individual plants that are connected by the parasitic plant *Cuscuta* can exchange systemic ROS, calcium, and electric signals in response to local stresses such as wounding or high light. As the transmission of systemic stress signals between different plants connected by a *Cuscuta* bridge could activate acclimation mechanisms and contribute to the survival of these plants (and/or the entire community) during stress, it is possible that plant interactions with *Cuscuta* may have some beneficial effects to plants, especially plants growing within a community. This possibility should be addressed in future studies as it may change the definition of *Cuscuta* from a parasitic plant to a partially mutualistic symbiosis.

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Author contributions

SP, YF, and RM conceived the ideas and designed the experiments. YF, AM and HL conducted the experiments and analyzed the data. SP, YF, and RM wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Conflicting interests

All authors declare that they have no conflicting interests.

152 **Figure Legends**

153 **Figure 1.** The experimental design used for measuring ROS accumulation following wounding.
154 **(a)** A *Cuscuta* bridge (white arrow) connected two *Arabidopsis* plants (donor *Arabidopsis* on the
155 left and receiver *Arabidopsis* on the right); stress was applied to a single leaf of the donor plant
156 (jagged yellow arrow). **(b)** Enlarged image of the red box in (a). The black arrow indicates
157 established haustorium developed on the *Arabidopsis* stem. **(c)** Representative 30 min time lapse
158 images of ROS accumulation in control plants or following mechanical wounding stress applied
159 to a single leaf of the donor plant. **(d)** Quantification of fluorescence corresponding to ROS content
160 at 0 min (grey) and after 30 min (white) in control treatment and following an injury of a single
161 leaf of the donor plants, in donor's local and systemic leaves, and in the receiver plant and *Cuscuta*
162 bridge. Asterisks indicated significance; Student's t test (*$P < 0.05$). Results are displayed as box-
163 and-whisker plots, with the borders corresponding to the 25th and 75th percentiles of the data.
164 Each data value is included as a point within each box plot, with the horizontal line representing
165 the median and "X" corresponding to the mean. Whiskers represent 1.5 times the minimum and
166 maximum of the mean (1.5 times of the interquartile range); size bar is 1 cm.

167 **Figure 2.** ROS accumulation and transcriptional changes in donor plant, *Cuscuta* bridge, and
168 receiver plant, in response to a local light stress applied to a single leaf of the donor plant.

169 **(a)** Representative images of ROS accumulation in control and high light stress-treated donor
170 plant, in a donor-*Cuscuta* bridge-receiver chain (light stress was applied to a single leaf of the
171 donor plant); **(b)** Quantification of fluorescence corresponding to ROS content at 0 min (grey) and
172 after 30 min (white) in control and following high light treatment applied to a single leaf of the
173 donor plants (local leaf), in *Arabidopsis* donor's local and systemic leaves, receiver and *Cuscuta*
174 plants; **(c, d)** Similar to (a) and (b), but for Ca^{2+} accumulation; **(e, f)** Similar to (a) and (b), but for
175 membrane potential changes; **(g)** Changes in the steady-state level of transcripts associated with
176 ROS responses in *Arabidopsis* donor and receiver (Rec) plants, measured under control conditions
177 (0 min; grey) or following treatment (30 min after light stress to a single leaf of the donor plant;
178 white); **(h)** Same as (g), but for *Cuscuta* ROS-response transcripts. Results are shown as box-and-
179 whisker plots, with the borders corresponding to the 25th and 75th percentiles of the data. Each
180 data value is included as a point within each box plot, with the horizontal line representing the
181 median and "X" corresponding to the mean. Whiskers represent 1.5 times the minimum and

182 maximum of the mean (1.5 times of the interquartile range). Quantitative real-time results are
183 presented as relative quantity PCR, normalized to reference gene. N=5-12, Asterisks indicated
184 significant difference; Student's t test (*P < 0.05, **P < 0.01 and ***P<0.001); size bar indicates
185 1 cm. Abbreviations: *APX1*, *ASCORBATE PEROXIDASE 1; CSD1, COPPER/ZINC*
186 *SUPEROXIDE DISMUTASE 1*; *GLR2.7, GLUTAMATE RECEPTOR 2.7; MYB30,*
187 *MYELOBLASTOSIS DOMAIN PROTEIN 30; NDPK2, NUCLEOSIDE DIPHOSPHATE KINASE*
188 *2;* Rec, Receiver; *ZAT10, ZINC FINGER OF ARABIDOPSIS THALIANA 10; ZAT12, ZINC*
189 *FINGER OF ARABIDOPSIS THALIANA 12; ZHD5, ZINC FINGER HOMEODOMAIN 5.*

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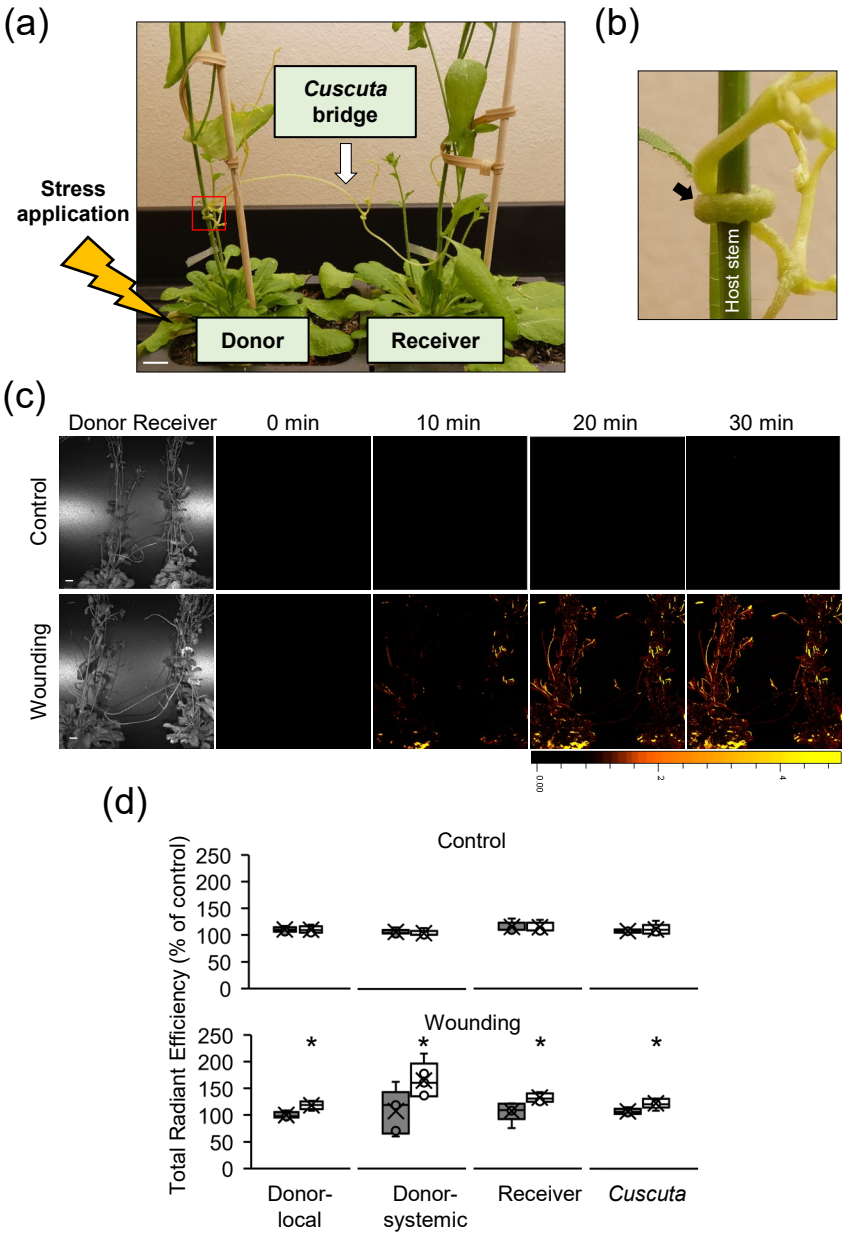

Figure 1. The experimental design used for measuring ROS accumulation following wounding. **(a)** A *Cuscuta* bridge (white arrow) connected two *Arabidopsis* plants (donor *Arabidopsis* on the left and receiver *Arabidopsis* on the right); stress was applied to a single leaf of the donor plant (jagged yellow arrow). **(b)** Enlarged image of the red box in (a). The black arrow indicates established haustorium developed on the *Arabidopsis* stem. **(c)** Representative 30 min time lapse images of ROS accumulation in control plants or following mechanical wounding stress applied to a single leaf of the donor plant. **(d)** Quantification of fluorescence corresponding to ROS content at 0 min (grey) and after 30 min (white) in control treatment and following an injury of a single leaf of the donor plants, in donor's local and systemic leaves, and in the receiver plant and *Cuscuta* bridge. Asterisks indicated significance; Student's t test (*$P < 0.05$). Results are displayed as box-and-whisker plots, with the borders corresponding to the 25th and 75th percentiles of the data. Each data value is included as a point within each box plot, with the horizontal line representing the median and "X" corresponding to the mean. Whiskers represent 1.5 times the minimum and maximum of the mean (1.5 times of the interquartile range); size bar is 1 cm.

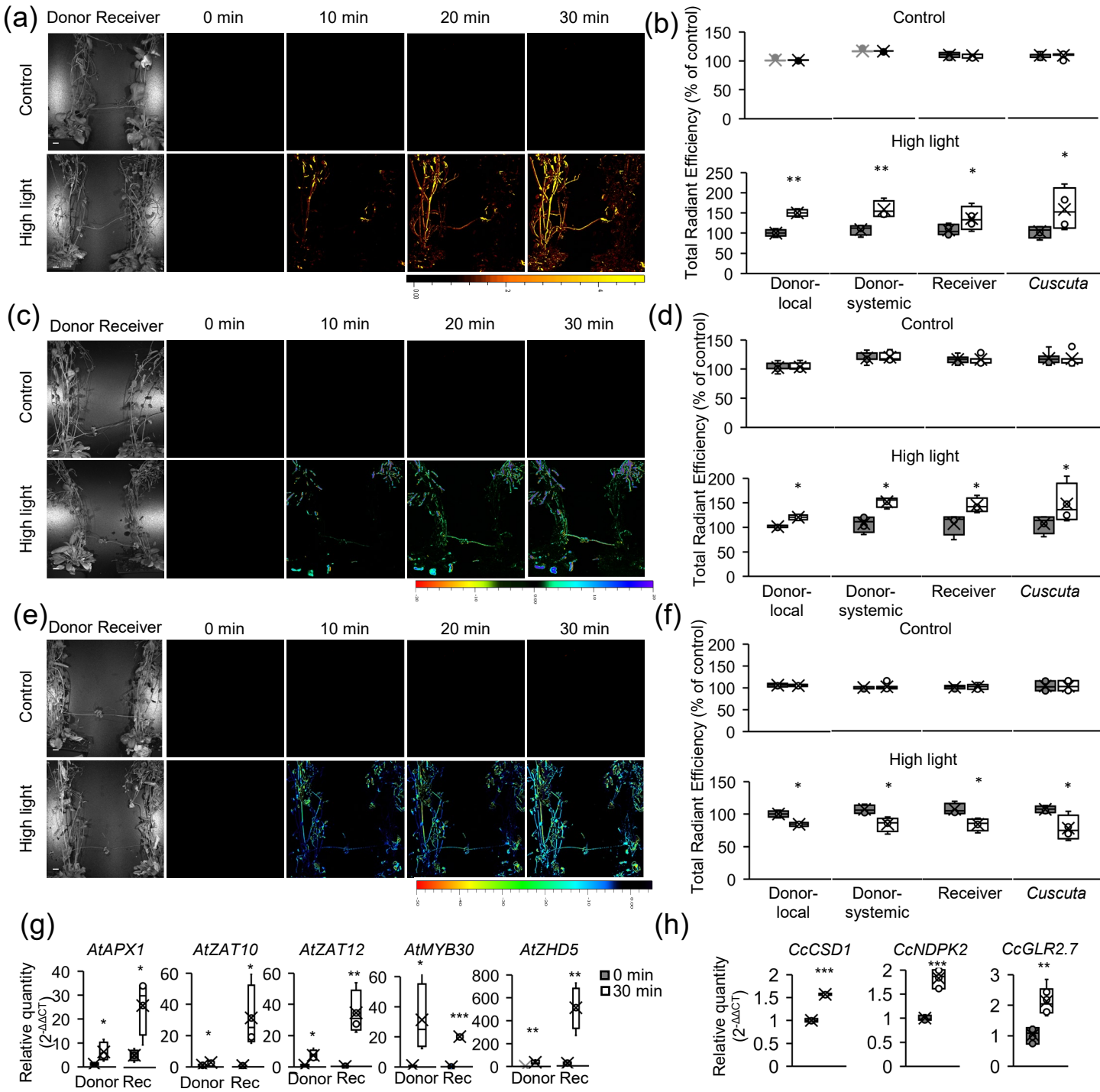

Figure 2. ROS accumulation and transcriptional changes in donor plant, *Cuscuta* bridge, and receiver plant, in response to a local light stress applied to a single leaf of the donor plant.

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