Calcium-Dependent Protein Kinase CPK6 Positively Functions in Induction by Yeast Elicitor of Stomatal Closure and Inhibition by Yeast Elicitor of Light-Induced Stomatal Opening in Arabidopsis[W][OPEN]

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Yeast elicitor (YEL) induces stomatal closure that is mediated by a Ca^{2+}-dependent signaling pathway. A Ca^{2+}-dependent protein kinase, CPK6, positively regulates activation of ion channels in abscisic acid and methyl jasmonate signaling, leading to stomatal closure in Arabidopsis (*Arabidopsis thaliana*). YEL also inhibits light-induced stomatal opening. However, it remains unknown whether CPK6 is involved in induction by YEL of stomatal closure or in inhibition by YEL of light-induced stomatal opening. In this study, we investigated the roles of CPK6 in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening in Arabidopsis. Disruption of *CPK6* gene impaired induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening. Activation by YEL of nonselective Ca^{2+}-permeable cation channels was impaired in *cpk6-2* guard cells, and transient elevations elicited by YEL in cytosolic-free Ca^{2+} concentration were suppressed in *cpk6-2* and *cpk6-1* guard cells. YEL activated slow anion channels in wild-type guard cells but not in *cpk6-2* or *cpk6-1* and inhibited inward-rectifying K^+ channels in wild-type guard cells but not in *cpk6-2* or *cpk6-1*. The *cpk6-2* and *cpk6-1* mutations inhibited YEL-induced hydrogen peroxide accumulation in guard cells and apoplast of rosette leaves but did not affect YEL-induced hydrogen peroxide production in the apoplast of rosette leaves. These results suggest that CPK6 positively functions in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening in Arabidopsis and is a convergent point of signaling pathways for stomatal closure in response to abiotic and biotic stress.

Stomata, formed by pairs of guard cells, play a critical role in regulation of plant CO_2 uptake and water loss, thus critically influencing plant growth and water stress responsiveness. Guard cells respond to a variety of abiotic and biotic stimuli, such as light, drought, and pathogen attack (Israelsson et al., 2006; Shimazaki et al., 2007; Melotto et al., 2008).

Elicitors derived from microbial surface mimic pathogen attack and induce stomatal closure in various plant species such as *Solanum lycopersicum* (Lee et al., 1999), *Commelina communis* (Lee et al., 1999), *Hordeum vulgare* (Koers et al., 2011), and Arabidopsis (*Arabidopsis thaliana*; Melotto et al., 2006; Khokon et al., 2010). Yeast elicitor (YEL) induces stomatal closure in Arabidopsis (Klüsener et al., 2002; Khokon et al., 2010; Salam et al., 2013). Our recent studies showed that YEL inhibits light-induced stomatal opening and that protein phosphorylation is involved in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening (Salam et al., 2013).

Cytosolic Ca^{2+} has long been recognized as a conserved second messenger in stomatal movement (Shimazaki et al., 2007; Roelfsema and Hedrich 2010; Hubbard et al., 2012). Elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is triggered by influx of Ca^{2+} from apoplast and release of Ca^{2+} from intracellular stores in guard cell signaling (Leckie et al., 1998; Hamilton et al., 2000; Pei et al., 2000; Garcia-Mata et al., 2003; Lemtiri-Chlieh et al., 2003). The influx of Ca^{2+} is carried by nonselective Ca^{2+}-permeable cation (I_{Ca}) channels that are activated by plasma membrane hyperpolarization and H_2O_2 (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). Elevation of $[Ca^{2+}]_{cyt}$ activates slow anion (S-type) channels and down-regulates inward-rectifying potassium (K_{in}) channels in guard cells (Schroeder and Hagiwara, 1989; Grabov and Blatt, 1999). The activation of S-type channels is a hallmark of stomatal closure, and the suppression of K_{in} channels is favorable to stomatal closure but not to stomatal opening (Pei et al., 1997; Kwak et al., 2001; Xue et al., 2011; Uraji et al., 2012).

YEL induces stomatal closure with extracellular H_2O_2 production, intracellular H_2O_2 accumulation, activation of I_{Ca} channels, and transient $[Ca^{2+}]_{cyt}$ elevations (Klüsener et al., 2002; Khokon et al., 2010). However, it remains to

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be clarified whether YEL activates S-type channels and inhibits K_{in} channels in guard cells.

Calcium-dependent protein kinases (CDPKs) are regulators in Ca^{2+}-dependent guard cell signaling (Mori et al., 2006; Zhu et al., 2007; Geiger et al., 2010, 2011; Zou et al., 2010; Munemasa et al., 2011; Brandt et al., 2012; Scherzer et al., 2012). In guard cells, CDPKs regulate activation of S-type and I_{Ca} channels and inhibition of K_{in} channels (Mori et al., 2006; Zou et al., 2010; Munemasa et al., 2011). A CDPK, CPK6, positively regulates activation of S-type channels and I_{Ca} channels without affecting H_2O_2 production in abscisic acid (ABA)- and methyl jasmonate (MeJA)-induced stomatal closure (Mori et al., 2006; Munemasa et al., 2011). CPK6 phosphorylates and activates SLOW ANION CHANNEL-ASSOCIATED1 expressed in *Xenopus* spp. oocyte (Brandt et al., 2012; Scherzer et al., 2012). These findings underline the role of CPK6 in regulation of ion channel activation and stomatal movement, leading us to test whether CPK6 regulates the induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening.

In this study, we investigated activation of S-type channels and inhibition of K_{in} channels by YEL and roles of CPK6 in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening. For this purpose, we examined the effects of mutation of *CPK6* on induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening, activation of I_{Ca} channels, transient $[Ca^{2+}]_{cyt}$ elevations, activation of S-type channels, inhibition of K_{in} channels, H_2O_2 production in leaves, and H_2O_2 accumulation in leaves and guard cells.

RESULTS

Impairment of Induction by YEL of Stomatal Closure and Inhibition by YEL of Light-Induced Stomatal Opening in *cpk6* Mutants

Application of 50 μg mL^{-1} YEL induced stomatal closure ($P < 0.001$; Fig. 1A) and inhibited light-induced stomatal opening ($P < 0.001$; Fig. 1B). YEL-induced stomatal closure was impaired in *cpk6-1* ($P = 0.60$) and *cpk6-2* ($P = 0.12$) mutants (Fig. 1A). Inhibition by YEL of light-induced stomatal opening was impaired in the *cpk6-1* ($P = 0.55$) and the *cpk6-2* ($P = 0.14$) mutants (Fig. 1B). These results suggest that CPK6 is involved in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening.

Impairment of Activation of I_{Ca} Currents and Induction of Transient $[Ca^{2+}]_{cyt}$ Elevations by YEL in *cpk6* Guard Cells

Application of 50 μg mL^{-1} YEL activated I_{Ca} currents in wild-type guard cell protoplasts (GCPs) ($P < 0.01$ at –180 mV; Fig. 2, A and C) but did not in the *cpk6-2* GCPs ($P = 0.37$ at –180 mV; Fig. 2, B and D). These results suggest that CPK6 is involved in activation of I_{Ca} channels by YEL.

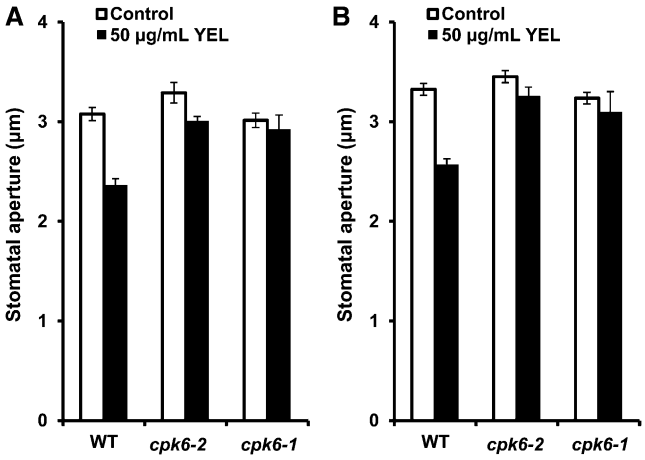

Figure 1. Induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening in the wild type and *cpk6-1* and *cpk6-2* mutants. A, YEL-induced stomatal closure in the wild type (WT) and *cpk6-1* and *cpk6-2* mutants. B, Inhibition by YEL of light-induced stomatal opening in the wild type and *cpk6-1* and *cpk6-2* mutants. Averages from three independent experiments (90 total stomata per bar) are shown. Error bars represent SES ($n = 3$).

In untreated wild-type plants, 30.9% of guard cells showed $[Ca^{2+}]_{cyt}$ elevations (Fig. 3C). Application of 50 μg mL^{-1} YEL induced $[Ca^{2+}]_{cyt}$ elevations in 96.2% of wild-type guard cells (Fig. 3, A and C). In untreated *cpk6-2* plants, 33.2% of guard cells showed $[Ca^{2+}]_{cyt}$ elevations (Fig. 3C). Application of 50 μg mL^{-1} YEL induced $[Ca^{2+}]_{cyt}$ elevations in 52.1% of *cpk6-2* mutant guard cells (Fig. 3, B and C), which is significantly lower than the percentage of number of guard cells showing $[Ca^{2+}]_{cyt}$ elevations in the wild type ($P < 0.01$). In untreated *cpk6-1* plants, 36.8% of guard cells showed $[Ca^{2+}]_{cyt}$ elevations (Supplemental Fig. S1). Application of 50 μg mL^{-1} YEL induced $[Ca^{2+}]_{cyt}$ elevations in 44.4% of *cpk6-1* mutant guard cells (Supplemental Fig. S1), which is significantly lower than the percentage of number of guard cells showing $[Ca^{2+}]_{cyt}$ elevations in the wild type ($P < 0.01$). These results suggest that CPK6 is involved in YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations.

Impairment of Activation of S-Type Currents and Suppression of K_{in} Currents by YEL in *cpk6* Guard Cells

Application of 50 μg mL^{-1} YEL activated S-type currents in wild-type GCPs ($P < 0.0001$ at –145 mV; Fig. 4, A and C) but did not in *cpk6-2* GCPs ($P = 0.12$ at –145 mV; Fig. 4, B and D) or *cpk6-1* GCPs (current = –16.26 ± 5.43 pA at –145 mV, $n = 3$; data not shown). YEL at 50 μg mL^{-1} suppressed K_{in} currents in wild-type GCPs ($P < 0.0001$ at –200 mV; Fig. 5, A and C) but did not in *cpk6-2* GCPs ($P = 0.15$ at –200 mV; Fig. 5, B and D) or *cpk6-1* GCPs (current = –290.75 ± 25.68 pA at –200 mV, $n = 4$; data not shown). These results suggest that CPK6 is involved in activation of S-type channels by YEL and suppression of K_{in} channels by YEL.

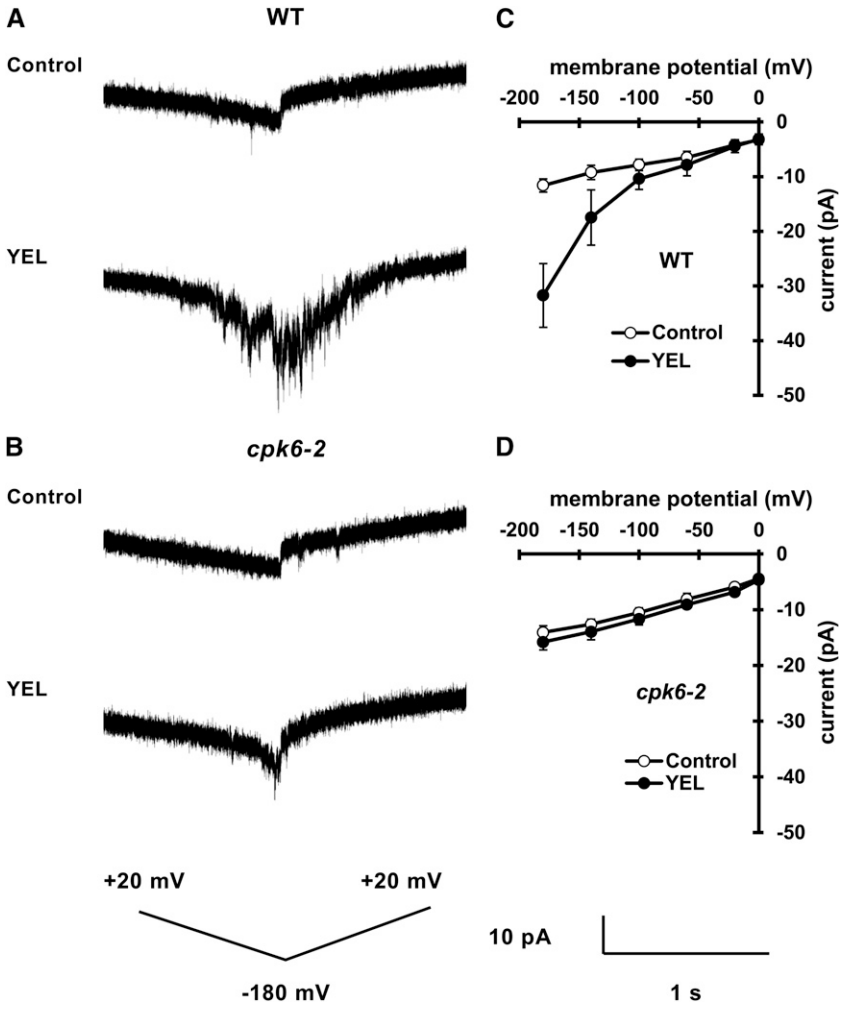

Figure 2. YEL activation of I_{Ca} currents in wild-type and *cpk6-2* GCPs. A, I_{Ca} currents in wild-type (WT) GCPs treated without YEL (top trace) or with 50 μg mL^{-1} YEL (bottom trace). B, I_{Ca} currents in *cpk6-2* GCPs treated without YEL (top trace) or with 50 μg mL^{-1} YEL (bottom trace). C, Current-voltage relationship for YEL activation of I_{Ca} currents in wild-type GCPs (n = 5) as recorded in A (white circles, control; black circles, 50 μg mL^{-1} YEL). D, Current-voltage relationship for YEL activation of I_{Ca} currents in *cpk6-2* GCPs (n = 5) as recorded in B (white circles, control; black circles, 50 μg mL^{-1} YEL). A ramp voltage protocol from +20 to –180 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was used. After making whole-cell configuration, GCPs were recorded 16 times to get averages for control. After adding YEL extracellularly, the GCPs were recorded for 16 times to get averages for the YEL treatment. The interpulse period was 1 min.

Effects of *cpk6* Mutations on YEL-Induced H_2O_2 Production and Accumulation

Application of 50 μg mL^{-1} YEL significantly induced H_2O_2 production in apoplast of wild-type, *cpk6-2*, and *cpk6-1* leaf tissues. There is no significant difference in YEL-induced apoplastic H_2O_2 production between the wild type and the *cpk6-2* mutant (P = 0.46 for total relative light units (RLU); P = 0.33 for max RLU; Fig. 6, A and B) and between the wild type and the *cpk6-1* mutant (P = 0.81 for total RLU; P = 0.36 for max RLU; Supplemental Fig. S2, A and B).

Application of 50 μg mL^{-1} YEL induced H_2O_2 accumulation in the apoplast of the wild-type leaf tissues ($P < 0.05$ at 20 min, Fig. 6C; $P < 0.05$ at 20 min, Supplemental Fig. S2C) but did not in the *cpk6-2* leaf tissues (P = 0.24 at 20 min, Fig. 6C) or the *cpk6-1* leaf tissues (P = 0.90 at 20 min, Supplemental Fig. S2C). Application of 50 μg mL^{-1} YEL induced H_2O_2 accumulation in wild-type guard cells ($P < 0.01$, Fig. 6D; $P < 0.05$, Supplemental Fig. S2D) but did not in the *cpk6-2* guard cells (P = 0.24, Fig. 6D) or the *cpk6-1* guard cells (P = 0.78, Supplemental Fig. S2D). These results suggest that CPK6 is involved in YEL-induced H_2O_2 accumulation in the apoplast of leaf tissues and in the cytoplasm of guard cells but not in H_2O_2 production.

DISCUSSION

CPK6 Is Involved in Induction by YEL of Stomatal Closure and Inhibition by YEL of Light-Induced Stomatal Opening

Our previous studies using the *cpk6-1* and *cpk6-2* mutants reveal that CPK6 positively regulates ABA- and MeJA-induced stomatal closure in Arabidopsis (Mori et al., 2006; Munemasa et al., 2011). In this study, the mutations of *CPK6* impaired the YEL-induced stomatal closure (Fig. 1). These results suggest that CPK6 is involved in YEL-induced stomatal closure and a key component shared with ABA- and MeJA-induced stomatal closure. Moreover, CPK3 functions in ABA-induced stomatal closure but not in MeJA- or YEL-induced stomatal closure (Mori et al., 2006; Munemasa et al., 2011; data not shown). Hence, CPK3 may function in stomatal response to drought stress rather than biotic stress, and regulation of CPK3 activation may be different from that of CPK6 in guard cells.

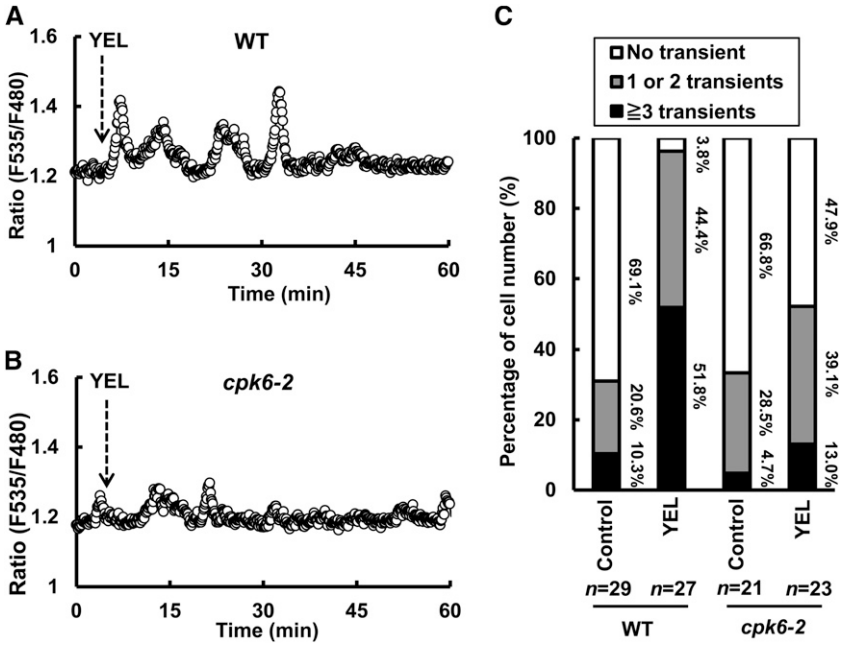

Figure 3. YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in wild-type and *cpk6-2* guard cells expressing YC3.6. A, A representative trace of fluorescence emission ratios (535/480 nm) showing 50 μg mL^{-1} YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in wild-type (WT) guard cells. B, A representative trace of fluorescence emission ratios (535/480 nm) showing 50 μg mL^{-1} YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in *cpk6-2* guard. C, Percentage of number of guard cells showing different number of transient $[Ca^{2+}]_{cyt}$ elevations in wild-type and *cpk6-2* guard cells. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in fluorescence emission ratios were more than or equal to 0.1 from the baseline.

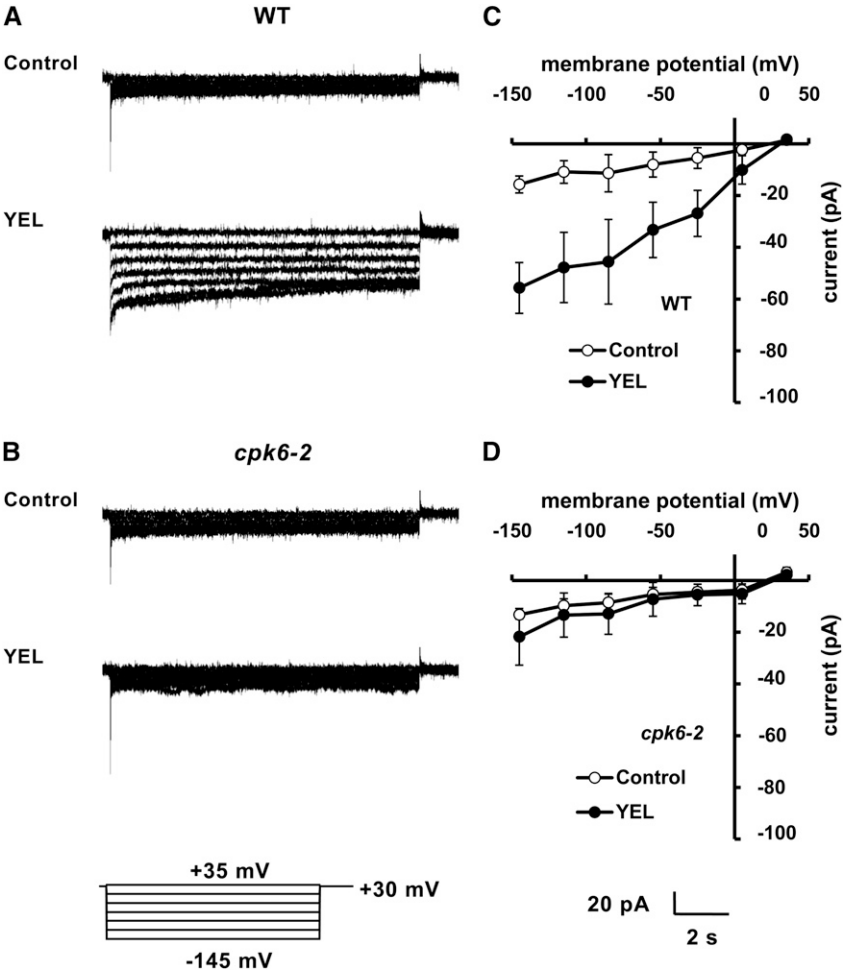

Figure 4. YEL activation of S-type currents in wild-type (WT) and *cpk6-2* GCPs. A, S-type currents in wild-type GCPs treated without (top trace) or with 50 μg mL^{-1} YEL (bottom trace). B, S-type currents in *cpk6-2* GCPs treated without (top trace) or with 50 μg mL^{-1} YEL (bottom trace). C, Steady-state current-voltage relationship for YEL activation of S-type currents in wild-type GCPs as recorded in A (white circles, control; black circles, YEL). D, Steady-state current-voltage relationship for YEL activation of S-type currents in *cpk6-2* GCPs as recorded in B (white circles, control; black circles, YEL). The voltage protocol was stepped up from +35 mV to –145 mV in 30-mV decrements (holding potential, +30 mV). GCPs were treated with YEL for 2 h before recordings. Each datum point was obtained from five GCPs. Error bars represent SES.

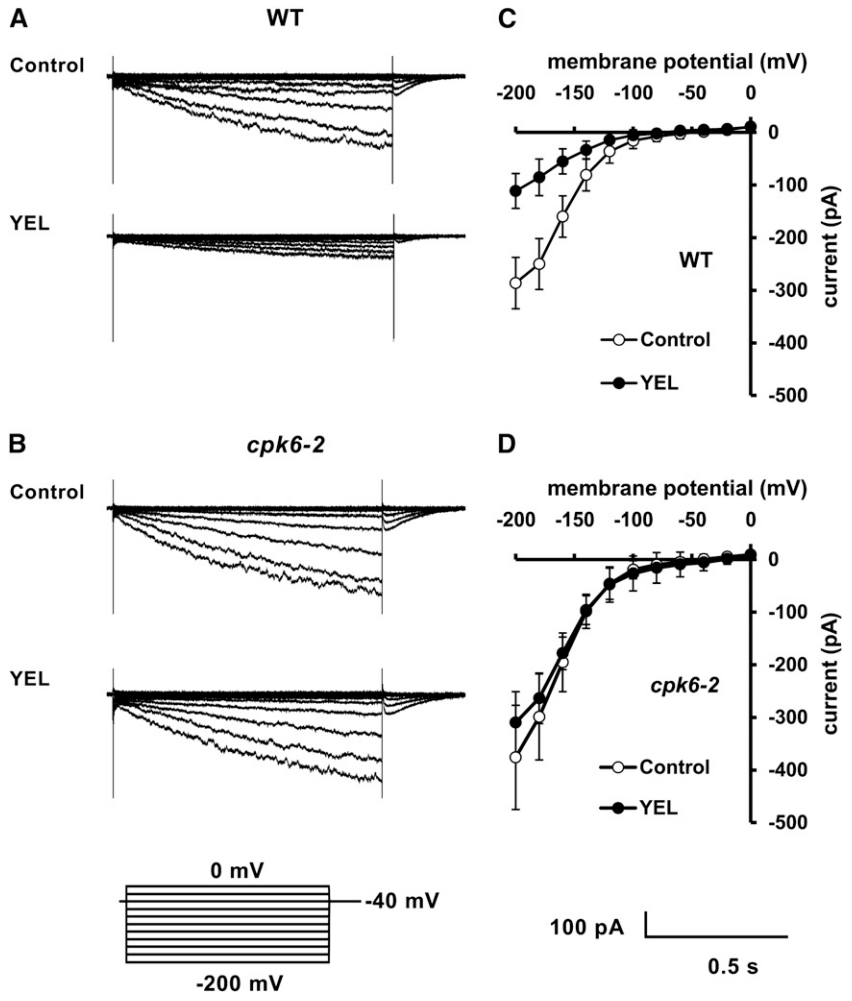

Figure 5. YEL inhibition of K_{in} currents in wild-type (WT) and *cpk6-2* GCPs. A, K_{in} currents in wild-type GCPs treated without (top trace) or with 50 μg mL^{-1} YEL (bottom trace). B, K_{in} currents in *cpk6-2* GCPs treated without (top trace) or with 50 μg mL^{-1} YEL (bottom trace). C, Steady-state current-voltage relationship for YEL inhibition of K_{in} currents in wild-type GCPs as recorded in A (white circles, control; black circles, YEL). D, Steady-state current-voltage relationship for YEL inhibition of K_{in} currents in *cpk6-2* GCPs as recorded in B (white circles, control; black circles, YEL). The voltage protocol was stepped up from 0 to –200 mV in 20-mV decrements (holding potential, –40 mV). GCPs were treated with YEL for 2 h before recordings. Each datum point was obtained from at least seven GCPs. Error bars represent SES.

It has been reported that CPK4, CPK10, and CPK11 function as positive regulators in inhibition by ABA of light-induced stomatal opening (Zhu et al., 2007; Zou et al., 2010). In this study, the mutations of *CPK6* impaired the inhibition by YEL of light-induced stomatal opening (Fig. 1), which suggests that CPK6 functions in YEL-induced inhibition of stomatal opening. Taken together, CPK6 may function as a key regulator in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening.

CPK6 Is Involved in YEL-Induced H_2O_2 Accumulation But Not in H_2O_2 Production

H_2O_2 functions as an important second messenger during stomatal closure (Pei et al., 2000; Zhang et al., 2001; Khokon et al., 2011; Salam et al., 2013). H_2O_2 activates I_{Ca} channels in guard cells, which triggers influx of Ca^{2+} through I_{Ca} channels from apoplast. Consequently, the influx of Ca^{2+} initiates transient elevations of $[Ca^{2+}]_{cyt}$ in guard cells (Pei et al., 2000; Allen et al., 2001; Murata et al., 2001). Releasing of Ca^{2+} from internal stores contributes to the following transient elevations of $[Ca^{2+}]_{cyt}$ (Garcia-Mata et al., 2003; Lemtiri-Chlieh et al., 2003).

H_2O_2 is mainly formed in the apoplast from superoxide produced by two NADPH oxidase catalytic subunits, AtrbohD and AtrbohF, in ABA and MeJA signaling (Kwak et al., 2003; Suhita et al., 2004; Jannat et al., 2011, 2012), and H_2O_2 is produced by apoplastic peroxidases in YEL signaling (Khokon et al., 2010), leading to the accumulation of H_2O_2 in guard cells. CPK6 is not involved in ABA- and MeJA-induced H_2O_2 accumulation in guard cells (Munemasa et al., 2011), whereas CPK6 is involved in YEL-induced H_2O_2 accumulation in guard cells but not in YEL-induced H_2O_2 production (Fig. 6, A and B; Supplemental Fig. S2, A and B). It appears that CPK6 is not involved in ABA-, MeJA-, or YEL-induced H_2O_2 production, but there is a difference in involvement of CPK6 between ABA-induced H_2O_2 accumulation and YEL-induced H_2O_2 accumulation, suggesting that there is a difference in the H_2O_2 scavenging system between ABA signaling and YEL signaling during stomatal closure.

In catalase mutants and catalase inhibitor-treated wild-type plants, ABA- and MeJA-induced stomatal closures are significantly enhanced, suggesting that H_2O_2 scavenging mechanisms are involved in stomatal closure

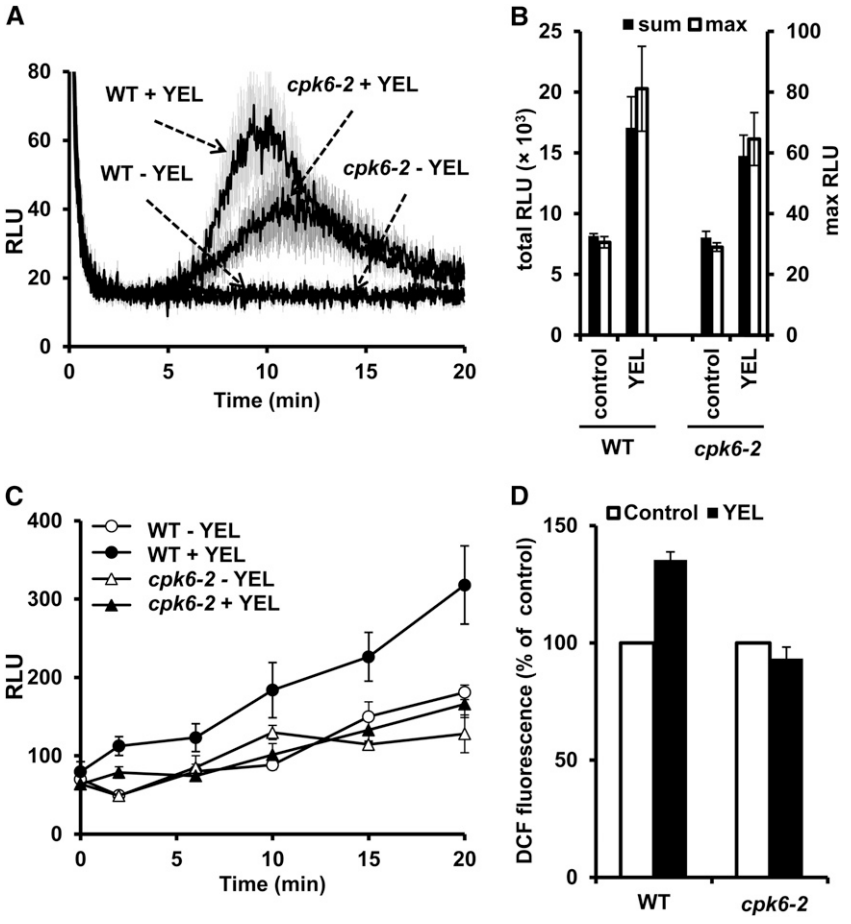

Figure 6. YEL-induced H_2O_2 production in apoplast of leaf tissues and H_2O_2 accumulation in apoplast of leaf tissues and guard cells. A, H_2O_2 production induced by 50 μg mL^{-1} YEL in apoplast of leaf tissues of the wild type (WT) and *cpk6-2* mutant. B, Total and maximal H_2O_2 production induced by YEL as recorded in A. The luminescence was measured between 1 and 20 min after adding YEL, where the luminescence at each sampling point was integrated for 2 s. "Total RLU" is the sum of luminescence between 1 and 20 min, and "max RLU" is the highest luminescence. C, H_2O_2 accumulation induced by 50 μg mL^{-1} YEL in the apoplast of leaf tissues of the wild type and *cpk6-2* mutant. D, H_2O_2 accumulation induced by 50 μg mL^{-1} YEL in guard cells of the wild type and *cpk6-2* mutant. H_2O_2 accumulation was expressed as the percentage of 2′,7′-dichlorofluorescein (DCF) fluorescence levels. Averages from three independent experiments (more than 150 total guard cells per bar in total) are shown.

(Jannat et al., 2011, 2012). An alcoholic yeast (*Saccharomyces cerevisiae*) extract does not have catalase activity but can accelerate activity of catalases and/or protect catalases from inactivation by H_2O_2 (Kreke et al., 1945; Sumner and Sisler, 1946; Kreke and Maloney, 1948). Therefore, CPK6 may negatively regulate the YEL-activated H_2O_2 scavenging system, including catalases. However, ABA does not significantly affect catalase activities (Jannat et al., 2011). This is why ABA induces H_2O_2 accumulation in the *cpk6* mutant as well as in the wild type.

CPK6 Positively Functions in Activation of I_{Ca} Channels and Transient $[Ca^{2+}]_{cyt}$ Elevations Induced by YEL

I_{Ca} channels are activated by H_2O_2, ABA, MeJA, and elicitors (Hamilton et al., 2000; Pei et al., 2000; Klüsener et al., 2002; Munemasa et al., 2007). The regulation of I_{Ca} channels also involves phosphorylation and $[Ca^{2+}]_{cyt}$ (Hamilton et al., 2000; Köhler and Blatt, 2002). CPK6 positively regulates the activation of I_{Ca} channels in ABA and MeJA signaling (Mori et al., 2006; Munemasa et al., 2011). In this study, YEL-induced I_{Ca} currents were impaired in the *cpk6-2* guard cells (Fig. 2). These results indicate that CPK6 is involved in activation of I_{Ca} channels induced by abiotic and biotic stimuli.

Activation by YEL of I_{Ca} channels required NADPH in the pipette solution (Klüsener et al., 2002; data not shown), and YEL-induced stomatal closure was not inhibited by an NADPH oxidase inhibitor, diphenylene iodonium chloride, or *atrbohD atrbohF* double mutation (Khokon et al., 2010). These results suggest that activation by YEL of I_{Ca} channels is modulated by redox status but not by activation of NADPH oxidases.

YEL-induced stomatal closure is accompanied by transient $[Ca^{2+}]_{cyt}$ elevations (Klüsener et al., 2002; Khokon et al., 2010; Salam et al., 2013; Fig. 3; Supplemental Fig. S1). In this study, mutations of *CPK6* impaired the YEL-induced $[Ca^{2+}]_{cyt}$ elevations (Fig. 3; Supplemental Fig. S1). The YEL-induced I_{Ca} currents were abolished, and the YEL-induced $[Ca^{2+}]_{cyt}$ elevations were impaired in the *cpk6-2* mutant. Hence, these results indicate that the impaired influx of Ca^{2+} from extracellular space contributes to the impaired $[Ca^{2+}]_{cyt}$ elevations.

CPK6 Positively Functions in Activation of S-Type Channels and Inhibition of K_{in} Channels Induced by YEL

Activation of S-type channels is essential for stomatal closure induced by various stimuli, such as H_2O_2, Ca^{2+}, ABA, MeJA, CO_2, and elicitors (Schroeder and Hagiwara,

1989; Pei et al., 1997; Munemasa et al., 2007; Koers et al., 2011; Xue et al., 2011; Hua et al., 2012). YEL was reported to activate anion channel in root cells (Wu et al., 2011). This study shows that YEL activated S-type channels in guard cells (Fig. 4). CPK6 positively regulates the activation of S-type channels in ABA and MeJA signaling (Mori et al., 2006; Munemasa et al., 2011). In this study, the activation by YEL of S-type currents was impaired by the *cpk6-2* mutation (Fig. 4) and *cpk6-1* mutation (data not shown), suggesting that CPK6 is a key component in activation of S-type channels induced by abiotic and biotic stimuli in Arabidopsis guard cells.

Inhibition of K_{in} channels is preferred in stomatal closure and impairs light-induced stomatal opening (Schroeder et al., 1987; Kwak et al., 2001; Khokon et al., 2011; Uraji et al., 2012). A 22-amino acid sequence of the conserved N-terminal part of flagellin, flg22, inhibits K_{in} channels in Arabidopsis guard cells (Zhang et al., 2008). In this study, YEL inhibited the K_{in} currents (Fig. 5). It was reported that CPK10 positively regulates the inhibition by ABA of K_{in} channels (Zou et al., 2010). The presented results showed that the *cpk6-2* (Fig. 5) and *cpk6-1* (data not shown) mutations impaired the inhibition by YEL of the K_{in} currents, suggesting CPK6 is a negative regulator of K_{in} channel activation.

ABA signaling is mediated by a calcium-dependent pathway and a calcium-independent pathway (Levchenko et al., 2005; Lee et al., 2009; Hubbard et al., 2012). YEL-induced stomatal closure is accompanied by transient $[Ca^{2+}]_{cyt}$ elevations (Klüsener et al., 2002; Khokon et al., 2010; Salam et al., 2013; Fig. 3; Supplemental Fig. S1), whereas flg22 induces stomatal closure, in which a Ca^{2+}-independent protein kinase, Open Stomata1, is involved (Melotto et al., 2006). Hence, YEL signaling, like ABA signaling, may employ a calcium-independent pathway.

CONCLUSION

The presented results suggest that CPK6 positively functions in induction by YEL of stomatal closure and inhibition by YEL of light-induced stomatal opening and is a convergent point of signaling pathways for stomatal closure in response to abiotic and biotic stress.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) wild type (ecotype Columbia), *cpk6-1* (SALK_093308), and *cpk6-2* (SALK_033392) were grown in pots containing a mixture of 70% (v/v) vermiculite (Asahi-Kogyo) and 30% (v/v) kureha soil (Kureha Chemical) in a growth chamber (photon flux density of 80 μmol m^{-2} s^{-1} under a 16-h-light/8-h-dark regime). The temperature and relative humidity in the growth chamber were 22°C ± 2°C and 60% ± 10%. Twice or three times a week, 0.1% (v/v) Hyponex solution was provided to the plants. $[Ca^{2+}]_{cyt}$ in guard cells was measured using a Ca^{2+}-sensing fluorescent protein, yellow cameleon3.6 (YC3.6) (Nagai et al., 2004). To obtain YC3.6-expressing *cpk6-2* mutant, *cpk6-2* mutants were crossed with wild-type plants that had previously been transformed with YC3.6.

Elicitor Preparation

YEL was prepared as described previously (Schumacher et al., 1987). Briefly, 200 g of commercial baker's yeast (*Saccharomyces cerevisiae*) was dissolved in 300 mL of 20 mM sodium citrate buffer (pH 7.0) and autoclaved at 121°C and 110,000 Pa for 60 min. The autoclaved suspension was centrifuged at 10,000*g* for 20 min, and the supernatant and ethanol were mixed in equal volume and stirred gently overnight. The mixture was centrifuged at 10,000*g* for 20 min, and the supernatant was mixed with 3 times of its volume of ethanol gently overnight. The precipitate obtained by decantation was lyophilized and stored at –80°C until use. Mass spectrometry analysis revealed that YEL at 50 μg mL^{-1} does not contain ABA, MeJA, or jasmonic acid at concentration higher than 1 μM (data not shown), and transcription analysis revealed that YEL at 50 μg mL^{-1} did not increase transcript levels of ABA-responsive gene *Responsive To Dessication29B* and jasmonic acid-responsive gene *Vegetative Storage Protein1* (data not shown). Note that YEL contains yeast mannan as a major biologically active compound (Schumacher et al., 1987).

Stomatal Aperture Measurement

Fully expanded young leaves from 4- to 5-week-old plants were excised for stomatal aperture measurements as described previously (Uraji et al., 2012). For assays of light-induced stomatal opening, leaves were floated on assay solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) with their adaxial surface upward in the dark for 2 h to close the stomata. After adding YEL (50 μg mL^{-1}), the leaves were kept in the light (80 μmol m^{-2} s^{-1}) for 3 h before measurement. For assays of stomatal closure, leaves were floated on the assay solution in the light for 2 h to open the stomata. Then YEL (50 μg mL^{-1}) was added, and the leaves were kept in the light for 2 h before measurement. For measurement of stomatal apertures, the leaves were shredded for 30 s, and epidermal tissues were collected using nylon mesh. Thirty stomatal apertures were measured for each sample.

Patch-Clamp Measurement

Current measurements of K_{in}, I_{Ca}, and S-type channels in Arabidopsis guard cells were performed as described previously (Mori et al., 2006; Uraji et al., 2012). Arabidopsis GCPs were prepared from rosette leaves of 4- to 6-week-old plants with the digestion solution containing 1.0% (w/v) Cellulase R10, 0.5% (w/v) Macerozyme R10, 0.5% (w/v) bovine serum albumin, 0.1% (w/v) kanamycin, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-mannitol (pH 5.5) with KOH. Whole-cell currents were recorded using a CEZ-2200 patch-clamp amplifier (Nihon Kohden). No leak subtraction was applied for all current-voltage curves. For data analysis, pCLAMP 10.3 software (Molecular Devices) was used.

For K_{in} channel current measurement, pipette solution contained 30 mM KCl, 70 mM K-Glu, 2 mM $MgCl_2$, 3.35 mM $CaCl_2$, 6.7 mM EGTA, and 10 mM HEPES-Tris (pH 7.1). Bath solution contained 30 mM KCl, 2 mM $MgCl_2$, 40 mM $CaCl_2$, and 10 mM MES-Tris (pH 5.5). For I_{Ca} channel current measurement, pipette solution contained 10 mM $BaCl_2$, 0.1 mM dithiothreitol, 5 mM NADPH, 4 mM EGTA, and 10 mM HEPES-Tris (pH 7.1). Bath solution contained 100 mM $BaCl_2$, 0.1 mM dithiothreitol, 1 mM $CaCl_2$, and 10 mM MES-Tris (pH 5.6). For S-type channel current measurement, pipette solution contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 5.58 mM $CaCl_2$ (free Ca^{2+} concentration, 2 μM), 5 mM ATP, and 10 mM HEPES-Tris (pH 7.1). Bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM MES-Tris (pH 5.6). In all cases, osmolarity was adjusted to 500 mmol kg^{-1} (pipette solutions) and 485 mmol kg^{-1} (bath solutions) with D-sorbitol.

Imaging of $[Ca^{2+}]_{cyt}$ in Guard Cells

Four- to 6-week-old wild-type and *cpk6-2* plants expressing YC3.6 were used for the measurement of $[Ca^{2+}]_{cyt}$ in guard cells as described previously (Uraji et al., 2012). The abaxial side of an excised leaf was gently mounted on a glass slide with a medical adhesive (stock no. 7730; Hollister), followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade to keep the lower epidermis intact on the slide. The remaining abaxial epidermis was incubated in solution containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) in the light for 2 h at 22°C to promote stomatal opening. Turgid guard cells were used to measure $[Ca^{2+}]_{cyt}$. Guard cells were treated with 50 μg mL^{-1} YEL using a peristaltic pump at 5 min after monitoring. For dual-emission ratio imaging of YC3.6, we used a 440AF21

excitation filter, a 445DRLP dichroic mirror, a 480DF30 emission filter for cyan fluorescent protein (CFP), and a 535DF25 emission filter for yellow fluorescent protein (YFP). The CFP and YFP fluorescence intensity of guard cells were imaged and analyzed using the W-View system and AQUA COSMOS software (Hamamatsu Photonics). CFP and YFP fluorescence were simultaneously monitored.

Detection of H_2O_2 in Apoplast of Leaf Tissues and Guard Cells

H_2O_2 produced in apoplast of leaf tissues was measured by a luminol-based assay (Trujillo et al., 2008). Leaf discs (3 mm in diameter) of 4- to 6-week-old plants were incubated in water for 16 h. H_2O_2 production was triggered by 100 μL reaction solution (100 μM luminol, 1 μg mL^{-1} horseradish peroxidase, and 50 μg mL^{-1} YEL). The luminescence was measured between 1 and 20 min after adding YEL, where the luminescence at each sampling point was integrated for 2 s, using a luminometer (AB2200, Atto). The experiments were repeated for seven times. Note that the assay using luminol monitors time course of production of H_2O_2 in extracellular space including cell wall.

We measured apoplastic H_2O_2 accumulation as the amount of H_2O_2 leaked from leaf tissues to 100 μL of a bathing solution. After incubated in water for 16 h, leaf discs were incubated in 100 μL water with or without YEL (50 μg mL^{-1}). At indicated time of treatment, H_2O_2 in the bathing solution, leaked from the leaf discs, was measured by a luminol-based assay. Briefly, 60 μL of the bathing solution was reacted with 40 μL of a reaction solution containing 50 μg mL^{-1} YEL, 250 μM luminol, and 2.5 μg mL^{-1} horseradish peroxidase. The luminescence was measured for 6 s. The experiments were repeated six times.

H_2O_2 accumulation in guard cells was evaluated using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Uraji et al., 2012). Epidermal tissues were isolated after leaves of 4- to 6-week-old plants shredded with a commercial blender. The epidermal tissues were incubated in medium containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) in the light at room temperature for 3 h. After incubation, 50 μM H_2DCF-DA was added to the medium. The epidermal tissues were incubated in the dark at room temperature for 30 min, and then the excess dye was washed out with the same medium. The dye-loaded tissues were treated with 50 μg mL^{-1} YEL in the dark at room temperature for 20 min. The stained guard cells were imaged using a fluorescence microscope (Biozero BZ-8000, Keyence) with an OP-66835 BZ GFP filter (excitation wavelength, 480/30 nm; absorption wavelength, 510 nm; and dichroic mirror wavelength, 505 nm). Fluorescence pictures of the guard cells were taken at optical magnification of ×40 with exposure time of 1.5 s. Excited light intensity was attenuated by a FM20 filter (transmission rate of 20%). The fluorescence levels of the guard cells were determined using ImageJ software (National Institutes of Health) and expressed as percentage of the control. Note that H_2DCF-DA permeates plasma membrane and is hydrolyzed by cytosolic esterases to yield a free form 2′,7′-dichlorodihydrofluorescein in cytosol and that 2′,7′-dichlorodihydrofluorescein can react with H_2O_2 in the presence of cytosolic peroxidases to produce the fluorescent oxidized form.

Statistical Analysis

The significance of differences between data sets was assessed by Student's t test. The response of $[Ca^{2+}]_{cyt}$ was assessed by χ^2 test. Differences were considered significant for $P < 0.05$.

Arabidopsis Genome Initiative number for the gene discussed in this article is as follows: *CPK6* (AT2G17290).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. YEL-induced transient $[Ca^{2+}]_{cyt}$ elevations in *cpk6-1* guard cells expressing YC3.6.

Supplemental Figure S2. YEL-induced H_2O_2 production in apoplast of leaf tissues and H_2O_2 accumulation in apoplast of leaf tissues and guard cells.

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