Reactive Oxygen Species and Gibberellin Acid Mutual Induction to Regulate Tobacco Seed Germination

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Seed germination is a complex process controlled by various mechanisms. To examine the potential contribution of reactive oxygen species (ROS) and gibberellin acid (GA) in regulating seed germination, diphenylene iodonium chloride (DPI) and uniconazole (Uni), as hydrogen peroxide ($H_2O_2$) and GA synthesis inhibitor, respectively, were exogenously applied on tobacco seeds using the seed priming method. Seed priming with DPI or Uni decreased germination percentage as compared with priming with $H_2O_2$, especially the DPI + Uni combination. $H_2O_2$ and GA completely reversed the inhibition caused by DPI or Uni. The germination percentages with $H_2O_2$ + Uni and GA + DPI combinations kept the same level as with $H_2O_2$. Meanwhile, GA or $H_2O_2$ increased GA content and deceased ABA content through corresponding gene expressions involving homeostasis and signal transduction. In addition, the activation of storage reserve mobilization and the enhancement of soluble sugar content and isocitrate lyase (ICL) activity were also induced by GA or $H_2O_2$. These results strongly suggested that $H_2O_2$ and GA were essential for tobacco seed germination and by downregulating the ABA/GA ratio and inducing reserve composition mobilization mutually promoted seed germination. Meanwhile, ICL activity was jointly enhanced by a lower ABA/GA ratio and a higher ROS concentration.

Keywords: tobacco seed, germination, $H_2O_2$, GA, ABA, homeostasis, signal transduction

INTRODUCTION

Seeds are important genetic resources for sustainable agriculture and agricultural economy. Successful germination and seedling establishment are the foundation of agricultural production (Bentsink and Koornneef, 2008). It has been proposed that seed germination is mediated by gibberellin acid (GA) that promotes germination completion. Abscisic acid (ABA) has antagonistic effects on GA in inducing seed dormancy. The dynamic balance of ABA and GA, resulting from their synthesis, catabolism, and signaling pathway, is crucial for seed germination (Razem et al., 2006; Seo et al., 2006; Weiss and Ori, 2007; Topham et al., 2017). Up to now, the molecular mechanism of interaction between ABA and GA during germination remains largely elusive. It is well known that NCED (encoding 9-cis-epoxycarotenoid dioxygenase) is a key gene in ABA biosynthesis, and CYP707A (encoding ABA 8-hydroxylases) is the essential gene in ABA catabolism (Footitt et al., 2011). ABA insensitive 3 (ABI3) and ABA insensitive 5 (ABI5), the central ABA signaling components, serve as the final downstream repressors of seed germination in the ABA signaling pathway (Carles et al., 2002; Lim et al., 2013; Liu et al., 2013). GA20ox (encoding...
GA 20-oxidase) and GA3ox (encoding GA 3-oxidase) are critical genes during bioactive GA biosynthesis and GA2ox (encoding GA 2-oxidase) transfers active GAs to inactive GAs. RGL2 (repressor of GA-like protein), one of plant growth repressor DELLAs (DELLAs) (Zentella et al., 2007; Goldack et al., 2013), is considered the vital repressor of seed germination (Piskurewicz et al., 2008; Stamm et al., 2012). Gibberellic-insensitive dwarf protein (GID), a receptor of GA signaling, could interact with GA and eliminate the inhibition of seed germination by triggering the degradation of RGL2 (Cao et al., 2006; Griffiths et al., 2006).

Seed reserve composition mobilization is an important requirement for germination as well as subsequent plant growth and development (Bau et al., 1997; Kupidłowska et al., 2006). It should be noted that the lipid content of tobacco seed is up to 40% by weight (Giannelos et al., 2002). Isocitrate lyase (ICL, EC 4.1.3.1) is a key enzyme during the glyoxylate cycle that plays a major role in affecting net gluconeogenesis from lipid metabolism (Borek and Nuc, 2011). Generally, seed germination and seedling establishment are restrained by inhibiting ICL activity, as (Borek and Nuc, 2011). Generally, seed germination and seedling establishment are restrained by inhibiting ICL activity, as demonstrated in oilseeds such as sunflower (Helianthus annuus L.), coffee (Coffeea arabica L.), and canola (Brassica napus L.) (Baleroni et al., 2000; Kupidłowska et al., 2006; Selmar et al., 2006). In addition, phytohormones ABA and GA are widely known to control the mobilization of lipids by inhibiting or inducing ICL activity and the responding gene expression in rice (Oryza sativa L.), wheat (Triticum aestivum L.), and barley (Hordeum vulgare L.) (Eastmond and Jones, 2005; Penfield et al., 2006).

Reactive oxygen species (ROS) act as regulators of growth and development, programmed cell death, hormone signaling, and responses to biotic and abiotic stresses (Breusegem et al., 2008). In seeds, ROS play a key role in various events, such as maturation, ripening, aging, and germination (Bailly et al., 2008; Müller et al., 2009; Barba-Espín et al., 2011). Plasma membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the pivotal enzyme involved in ROS generation, regulates germination of barley seeds (Ishibashi et al., 2010). Hydrogen peroxide (H$_2$O$_2$) is considered as the ROS messenger for long-distance transport in cells (Moller, 2001; Möller and Sweetlove, 2010), the stimulation of downstream transcription, and the activation of mitogen-activated protein kinases (MAPKs) (Neill S. et al., 2002). Exogenous H$_2$O$_2$ stimulates seed germination of Arabidopsis (Arabidopsis thaliana L.), maize (Zea mays L.), and sunflower seeds by enhancing GA biosynthesis and ABA catabolism (Maraouf et al., 2015; Huang et al., 2017; Li et al., 2017). Nevertheless, H$_2$O$_2$ releases embryo dormancy in barley by activating GA signaling and synthesis rather than the repression of ABA signaling (Bahin et al., 2011). GA biosynthesizes in embryo during the germination and induces H$_2$O$_2$ production in wheat aleurone cells; H$_2$O$_2$ subsequently acts as a signal molecule to antagonize ABA signaling (Wu et al., 2014). Additional evidence has suggested that GA induces the germination of dormant caryopses by regulating the level of ABA and the ROS-antioxidant state (Cembrowska-Lech et al., 2015). Programmed cell death is induced by GA in aleurone layers also via the regulation of H$_2$O$_2$ production (Aoki et al., 2014). The application of H$_2$O$_2$-induced cell death was observed only under GA treatment but not ABA treatment (Bailly, 2013). Although several evidences indicate the crosstalk among ROS, GA and ABA affect seed germination, but the interaction between them remains elusive. Is there a relationship between GA and ROS that mutually induced or maintained homeostasis during seed germination? Similarly, it is unknown how ROS and GA synergistically control ABA levels during seed germination. Moreover, the relationship among ROS, GA, and ABA with ICL activity also has not been analyzed in tobacco seed germination. Therefore, the purpose of the present study is to examine the interaction mechanism of H$_2$O$_2$, GA, ABA, and ICL in the process of germination. The effect of diphenylene iodonium chloride (DPI), a NADPH oxidases inhibitor (Morré, 2002), uniconazole (Uni), a GA synthesis inhibitor (Yang et al., 2012), DPI + GA and Uni + H$_2$O$_2$ on seed germination is investigated. In addition, ICL activity, ABA, GA, H$_2$O$_2$ contents, and the corresponding gene expressions involved in homeostasis and signal transduction are determined to gain insights on the crosstalk among ROS, ABA, and GA, and to acquire the regulation mechanism of ICL activity, by proposing a comprehensive model of tobacco seed germination.

**MATERIALS AND METHODS**

**Materials**

Tobacco seeds, MS Yunyan 97, from Yunnan Tobacco Science Research Institute were used. GA, H$_2$O$_2$, ABA, and DPI were obtained from Aladdin Company, Hangzhou, China.

**Seed Priming**

Seeds were surface-sterilized with 0.5% NaClO solution for 15 min and then washed three times with sterilized distilled water to remove the residue of the disinfectant. The sterilized seeds were poured into a sealed beaker with priming solutions (1:5, w/v) at 20°C in darkness for 24 h, without changing the solutions during the priming process. All primed seeds were air-dried at 25°C for 48 h to the original moisture content. In this study, ten priming solutions including distilled water (H$_2$O$_2$), 10 mM GA, 50 mM H$_2$O$_2$, 30 μM Uni, 100 μM DPI, and the corresponding combination priming GA + DPI, H$_2$O$_2$ + Uni, GA + Uni, H$_2$O$_2$ + DPI, and Uni + DPI were all carried out.

**Seed Germination**

All primed tobacco seeds were germinated on three-layer water-saturated filter papers in germination dishes of 10 cm diameter at 25°C, with a photosynthetic active photon flux density of 250 mmol m$^{-2}$ s$^{-1}$ and a photosperiod of 8 h light (L): 16 h dark (D). In addition, four replications of 100 seeds for each treatment were used. Germinated seeds (radicle emergence) were recorded daily (International Seed Testing Association [ISTA], 2010). Germination percentage (GP) was calculated from the 1st to the 16th day (GP = 100% × Gt/100, where Gt is the total germinated seeds on the tth day. Germination...
energy (GE) was the GP on the 7th day. Germination index (GI) and mean germination time (MGT) were also calculated on the 2nd, 5th, and 7th days \[GI = \sum (G_t/T_t); \quad MGT = \sum (G_t \times T_t)/\sum G_t, \quad \text{where } G_t \text{ is the number of new germinated seeds in time } T_t.\]

**ICL Activity Determination**

The ICL activity assay was performed as described by Yanik and Donaldson (2005). The mixture containing 72.5 mM phosphate buffer (pH 6.9), 10.8 mM MgCl$_2$, and 10 mM phenylhydrazine in a volume of 1 ml was used. Freshly prepared 1.74 mM isocitrate and protein extracts were then added at 25°C. Phenylhydrazine forms a yellow phenylhydrazone in the presence of glyoxylic acid. The activity was detected by the increase in absorbance at 324 nm using the molar extinction coefficient $\varepsilon = 1.7 \times 10^4$.

**H$_2$O$_2$ and Soluble Sugar Content Measurements**

Seeds were collected at 0, 24, and 48 h during imbibition. For each treatment, 0.4 g seeds with four biological replications were collected for physiological parameter determination. H$_2$O$_2$ content was determined according to the method of Douli et al. (1997), and calculated as µmol H$_2$O$_2$ decomposition min$^{-1}$ g$^{-1}$ FW. Soluble sugar content in seeds was measured by using the anthrone colorimetric method (Li and Li, 2013) with a slight modification.

**ABA and GA$_3$ Measurements**

Seeds were collected at 0, 24, and 48 h during imbibition. Approximately 0.4 g seed powder for each treatment with three biological replications were homogenized with 2 mL of cold...
acetonitrile, kept at 4°C for 12 h and then centrifuged at 23,000 × g for 10 min at 4°C. The supernatant was transferred to a new centrifugal tube and mixed with 1.5 mL of phosphate buffer solution (0.1 M), kept at −80°C for 30 min and thawed adequately at 4°C. The mixture was extracted by 2.5 mL of ethyl acetate three times after the addition of 1 mL hydrochloric acid (0.5 mM). Five milliliters of the ethyl acetate phase was collected after centrifuging at 1500 × g for 5 min at 4°C, dried with a nitrogen evaporator, and redissolved in 1 mL mobile phase consisting of methanol and water (1:1, v/v). The collected extract was filtered through a 0.22 mm membrane filter for high-performance liquid chromatography equipped with a 6.0 mm × 150 mm, a 5 mm particle size reverse-phase (C18) column, and an ultraviolet detector (Waters 2487), as previously

FIGURE 3 | ICL activity decreased accompanied by seed imbibition. (A,B) ICL, isocitrate lyase. Seeds were collected, respectively, at 0, 24, and 48 h during imbibition, and four replications for each treatment at each sampling time were used. Different small letters on top of the bars indicate significant differences (LSD, α = 0.05) among treatments at the same imbibition time. Error bars indicate ± SE of mean (n = 4). For additional explanations, please see Figure 1.

FIGURE 4 | Inhibitor of GA or H₂O₂ increased ABA (A,B) and GA₃ (C,D) content during seed imbibition in response to different treatments. Seeds were collected, respectively, at 0, 24, and 48 h during imbibition, and four replications for each treatment at each sampling time were used. Different small letters on top of the bars indicate significant differences (LSD, α = 0.05) among treatments at the same imbibition time. Error bars indicate ± SE of mean (n = 4). For additional explanations, please see Figure 1.
described (Huang et al., 2017). GA₃ and ABA peaks were detected by using a SPD-20A (Shimadzu) absorbance detector at 254 nm. The mobile phase consisted of methanol and 0.2% phosphoric acid solution (1:1, v/v) at a flow rate of 0.8 mL min⁻¹. Standard GA₃ and ABA (Sigma Chemical Company) were used for the development of standard curves.

### Total RNA Extraction and Quantitative Real-time PCR (RT-qPCR) Analysis

Total RNA was isolated from seeds using TRIzol reagent (Huayueyang, Beijing, China) and reverse-transcribed using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) following the manufacturer’s instructions. Eight genes were involved in the tobacco GA signal: NtGA20ox1, NtGA20ox2, NtGA3ox2, NtGA2ox1, NtGA2ox2, NtGID1, NtGID2, and NtRGL2. Four genes were involved in the ABA signal: NtNCED1, NtNCED3, NtCYP707A1, and NtCYP707A2. NtRBOH (Nicotiana tabacum respiratory burst oxidase homolog) and NtICL were involved in NADPH oxidase and ICL biosynthesis (Supplementary Table S1). RT-qPCR was performed using the Roche real-time PCR detection system (Roche Life Science, United States). Each reaction (20 µL) consisted of 10 µL SYBR Green PCR Master Mix (Takara, Chiga, Japan), 1 µL diluted cDNA, and 0.1 µM forward and reserve primers. The PCR cycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 58°C for 45 s. The tobacco Actin gene was used as an internal control. Relative gene expression was calculated according to Livak and Schmittgen (2001).

### Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS) (version 9.2) followed by the calculation of the least significant difference (LSD, α = 0.05). Percentage data were arc-sin-transformed prior to analysis.

## RESULTS

### Uni or DPI Significantly Slowed Down Germination Speed and Reduced GP

Uni or DPI significantly inhibited seed germination compared with other treatments (Figure 1 and Supplementary Figure S2). The GP of Uni was 35% ultimately, and that of DPI was 43%. Seeds treated by Uni + DPI barely germinated, and GP was only 5% until the end of germination (Supplementary Figure S1). The seed germination inhibition of Uni or DPI could be reversed by adding GA or H₂O₂. Eventually, the GP of H₂O₂ + DPI and GA + Uni reached 92%, respectively (Supplementary Figure S1). Interestingly, the combination of H₂O₂ + Uni or GA + DPI reversed the inhibition effect of Uni or DPI on germination,

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**FIGURE 5 |** GA or H₂O₂ decreased the transcript level of ABA biosynthesis genes NtNCED1 (A,B) and NtNCED3 (C,D) during seed imbibition. Seeds were collected, respectively, at 0, 24, and 48 h during imbibition, and four replications for each treatment at each sampling time were used. Different small letters on top of the bars indicate significant differences (LSD, α = 0.05) among treatments at the same imbibition time. Error bars indicate ± SE of mean (n = 4). For additional explanations, please see Figure 1.
and the GPs of both were greater than 90%. Seeds treated with 
H$_2$O$_2$ or GA started germinating on the second day, and those 
treated with H$_2$O$_2$ + Uni or GA + DPI began to germinate on 
the third day, followed by H$_2$O on the fourth day, while seeds 
treated with Uni or DPI delayed germination until the sixth day 
of germination when the GP of GA or H$_2$O$_2$ had already reached 
92% (Figure 1 and Supplementary Table S2).

GA and H$_2$O$_2$ Treatments Increased 
Endogenous H$_2$O$_2$ Content During Seed 
Imbibition

Exogenous H$_2$O$_2$ and GA obviously increased endogenous H$_2$O$_2$ 
content, while DPI and Uni significantly reduced the content 
at 0 h of imbibition (also the end time of priming) compared 
with H$_2$O (Figure 2). GA + DPI reversed the reduction of 
endogenous H$_2$O$_2$ caused by DPI, and H$_2$O$_2$ + Uni also reversed 
the reduction caused by Uni, especially from 0 to 24 h.

ICL Activity and Soluble Sugar Content 
Decreased Accompanied by Seed 
Imbibition

ICL activity had an observable decrease at 48 h that was about 
half the ICL activity at 0 h (Figure 3). The ICL activity of DPI 
and Uni was notably lower than that of H$_2$O at 0 and 24 h, and 
at 24 h, respectively. The maximal ICL activity was recorded in 
H$_2$O$_2$ or GA, followed by H$_2$O$_2$ + Uni or GA + DPI from 0 to 
24 h. Ultimately, all treatments kept the same level at 48 h.

The change trend of soluble sugar was consistent with ICL 
activity. The main difference was that soluble sugar content 
dropped significantly at 24 h, which was earlier than 48 h when 
the changes in ICL activity occurred (Supplementary Figure S4). 
H$_2$O$_2$ or GA increased the content of soluble sugar at 0 and 
24 h, significantly at 24 h, while Uni or DPI decreased the 
content at 0 and 48 h, significantly at 48 h. H$_2$O$_2$ + Uni or 
GA + DPI slightly reversed the effects induced by Uni or DPI 
at 0 and 48 h.

Fluctuation of Endogenous ABA and GA$_3$ 
Content During Imbibition

It was rather obvious that both Uni and DPI dramatically induced 
endogenous ABA accumulation at the beginning of imbibition 
(Figures 4A,B, 0 h). GA decreased ABA content throughout the 
whole imbibition period, while H$_2$O$_2$ decreased ABA content at 
0 and 24 h. In general, H$_2$O$_2$ + Uni or GA + DPI counteracted 
the accumulation of ABA induced by Uni or DPI, of which ABA 
content was still higher than that in GA or H$_2$O$_2$, especially at 0 h.

In addition, the rapid and extensive generation of endogenous 
GA$_3$ with H$_2$O$_2$ or GA treatment was also significantly higher 
than that with H$_2$O treatment at 0 and 24 h. Uni markedly 
decreased GA$_3$ content, which was distinctly lower than H$_2$O 
from 0 to 48 h (Figure 4C). H$_2$O$_2$ + Uni obviously alleviated

**FIGURE 6** | GA or H$_2$O$_2$ enhanced the transcript level of ABA catabolism genes NtCYP707A1 (A,B) and NtCYP707A2 (C,D) during seed imbibition. Seeds were 
collected, respectively, at 0, 24, and 48 h during imbibition, and four replications for each treatment at each sampling time were used. Different small letters on top of 
the bars indicate significant differences (LSD, $\alpha = 0.05$) among treatments at the same imbibition time. Error bars indicate ± SE of mean ($n = 4$). For additional 
explanations, please see Figure 1.
the decrease in GA₃ content caused by Uni at 0 and 24 h and stabilized at the same GA₃ level as H₂O at 48 h. GA₃ significantly declined at 48 h with DPI treatment (Figure 4D). Exogenous GA obviously increased GA₃ content at 0 and 24 h. GA + DPI slightly decreased GA₃ content comparing with exogenous GA, which was still significantly higher than H₂O from 0 to 24 h.

The ABA/GA ratio of Uni and DPI was obviously higher than other treatments at 0 h, which was 2–3 fold as much as H₂O (Supplementary Figure S3), and then decreased sharply at 24 h, when Uni was still significantly higher than H₂O. The ABA/GA ratio and H₂O remained at lower levels during the whole imbibition process.

ABA Homeostasis and Signal Transduction Were Affected in Different Treatments During Imbibition

Both Uni and DPI induced the expression of NtNCED1 and NtNCED3, ABA synthesis genes, at the end of priming (Figure 5, 0 h). The NtNCED1 expression of Uni was 6-fold of H₂O, and that of DPI was 2.5-fold. Meanwhile, the NtNCED3 transcription level of DPI was 3.2-fold of H₂O, and that of Uni was 2.5-fold. H₂O₂ significantly decreased NtNCED3 expression and slightly decreased NtNCED1 expression, while GA significantly decreased NtNCED1 expression. At 48 h, H₂O₂ + Uni significantly decreased the expression of NtNCED3, while GA + DPI decreased the expression of both NtNCED1 and NtNCED3.

Gibberellin acid dramatically promoted the transcription level of NtCYP707A1 and NtCYP707A2, ABA catabolism genes, which was, respectively, 12-fold and 17-fold of H₂O, followed by GA + DPI with 9- and 14-fold of H₂O, while the effect of H₂O₂ was weaker than GA, approximately 3.6- and 4-fold of H₂O (Figure 6). Uni significantly decreased the expression of NtCYP707A1 and NtCYP707A2 from 0 to 48 h and at 48 h, respectively, and both remained at a low level during imbibition. DPI had no obvious function on the expression of NtCYP707A1 and NtCYP707A2.

In addition, Uni significantly enhanced NtABI3 and NtABI5 expression compared with H₂O during the whole process of imbibition (Figure 7), and DPI upregulated the expression of NtABI3 from 0 to 24 h and NtABI5 from 24 to 48 h. The downregulation induced by H₂O₂ was not obvious in NtABI3 and NtABI5 similarly to H₂O₂ + Uni. GA downregulated the expression of NtABI3 from 0 to 24 h as well as the expression of NtABI5 from 24 to 48 h, consistent with GA + DPI.

GA Homeostasis and Signal Transduction Were Mediated in Different Treatments During Imbibition

H₂O₂ dramatically enhanced the transcription level of GA synthesis genes (NtGA20ox1, NtGA20ox2, and NtGA3ox2). GA only obviously increased the expression of NtGA20ox2 (Figure 8). Uni largely inhibited NtGA20ox2 expression at 0

![Figure 7](link)
and 48 h, while \( \text{H}_2\text{O}_2 \) + Uni significantly upregulated the expression of \( \text{NtGA20OX2} \) from 0 to 24 h, \( \text{NtGA20OX1} \) at 24 h, and \( \text{NtGA3OX2} \) at 0 h, respectively. In addition, DPI mainly inhibited the expression of \( \text{NtGA3OX2} \) and was ineffective in \( \text{NtGA20OX1} \) and \( \text{NtGA20OX2} \). GA + DPI obviously upregulated the expression of \( \text{NtGA20OX2} \), followed by \( \text{NtGA20OX1} \) and \( \text{NtGA3OX2} \).

The transcription level of the GA catabolism gene \( \text{NtGA2OX1} \) was significantly decreased by \( \text{H}_2\text{O}_2 \) at 24 h, but not found in exogenous GA treatment (Figure 9). There was no significant difference among GA, \( \text{H}_2\text{O}_2 \), and \( \text{H}_2\text{O} \) treatments with regard to \( \text{NtGA2OX2} \). The expression of the two \( \text{NtGA2OX} \) genes was increased rapidly under the Uni condition, and was notably reversed by the addition of \( \text{H}_2\text{O}_2 \), meanwhile, \( \text{NtGA2OX1} \) expression was upregulated by DPI during the entire imbibition period but inhibited by GA + DPI.

Uni significantly upregulated the transcription level of \( \text{NiRGL2} \) and downregulated the \( \text{NiGID2} \) gene expression, while DPI only influenced the upregulation of \( \text{NiRGL2} \) (Figure 10). \( \text{H}_2\text{O}_2 \) enhanced the expression of \( \text{NiGID1} \) and \( \text{NiGID2} \) as did \( \text{H}_2\text{O}_2 \) + Uni, significantly at 0 h. GA also downregulated the \( \text{NiRGL2} \) expression and upregulated \( \text{NiGID1} \) and \( \text{NiGID2} \); especially, the \( \text{NiGID1} \) expression was 40-fold of \( \text{H}_2\text{O} \) at 0 h. GA + DPI obviously enhanced the expression of \( \text{NiGID1} \) and \( \text{NiGID2} \) during the whole imbibition process.

**Treatments Modulated the Expression of \( \text{NtRBOH} \) and \( \text{NtICL} \) During Imbibition**

The transcription of NADPH oxidase gene (\( \text{NtRBOH} \)), involved in \( \text{H}_2\text{O}_2 \) production, was markedly upregulated by priming with GA (Figure 11), and was reversed by the addition of DPI in
GA + DPI. Conversely, there was only weak enhancement or decrease with H$_2$O$_2$ or Uni compared with H$_2$O, as well as H$_2$O$_2$ + Uni. NtICL was obviously upregulated by exogenous H$_2$O$_2$ or GA, especially H$_2$O$_2$ treatment, the NtICL expression was 10-fold of H$_2$O at 0 h. Meanwhile, Uni kept the same level as H$_2$O but DPI was significantly lower than H$_2$O at the end of priming (Figure 11D, 0 h). The expression of NtICL in H$_2$O$_2$ + Uni was significantly higher than in H$_2$O at 0 h and 24 h, and it was not found in GA + DPI.

**DISCUSSION**

Gibberellin acid was crucial for seed germination, and the seed germination ability was also related to the accumulation of H$_2$O$_2$ to a critical level (Weiss and Ori, 2007; Huang et al., 2017). In the present study, H$_2$O$_2$ and GA accelerated seeds to germinate 2 days in advance. While DPI and Uni, which are considered as generation inhibitors of H$_2$O$_2$ and GA, respectively, significantly delayed the germination by 2 and 1 days, respectively (Figure 1 and Supplementary Figure S2). Meanwhile, the GP of DPI and Uni was obviously decreased, whereas the GP of DPI (43%) was slightly higher than that of Uni (35%). These results suggest that both GA and H$_2$O$_2$ are essential for seed germination and to guarantee germination speed and percentage. Similar results were found in flixweed (*Descurainia sophia* L.) and rice seeds; 10 µM uniconazole completely inhibited flixweed seed germination, and rice seed germination was inhibited by 10 µM DPI (Li et al., 2005; Ye and Zhang, 2012). Further evidence was presented in DPI + Uni treatment in which seeds barely germinated during the whole germination progress (Supplementary Figure S1). In addition, with H$_2$O$_2$ + Uni or GA + DPI treatment, seeds germinated 1 day in advance and the GP reached the same high level as with H$_2$O$_2$ + DPI or GA + Uni (Supplementary Figure S1). Furthermore, exogenous GA significantly increased the endogenous H$_2$O$_2$ content but Uni inhibited H$_2$O$_2$ synthesis (Figure 2). Likewise, exogenous H$_2$O$_2$ induced endogenous GA synthesis but DPI decreased the GA content (Figures 4C,D). These results all led to the preliminary conclusion that GA and H$_2$O$_2$ may mutually induce and have a synergistic effect on seed germination.

It is well known that ABA and GA are essential regulation factors in seed dormancy and germination (Toh et al., 2008; Stamm et al., 2012; Maarouf et al., 2015), and the antagonistic crosstalk between GA and ABA plays a pivotal role in the modulation of seed germination (Piskurewicz et al., 2008; Liu et al., 2016). Studies on numerous species have demonstrated that H$_2$O$_2$ promotes seed germination by triggering GA biosynthesis and ABA catabolism (Liu et al., 2010). DPI or Uni priming obviously increased the ABA content and the ABA/GA ratio (Figures 4A,B and Supplementary Figure S3), and decreased endogenous H$_2$O$_2$ content at the end of priming (Figure 2). These may be the reasons for intense reduction in the germination speed and the GP resulting from DPI or Uni.

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**FIGURE 9** | GA or H$_2$O$_2$ mediated the transcript level of GA catabolism genes NtGA2ox1 (A,B) and NtGA2ox2 (C,D) during seed imbibition. Seeds were collected, respectively, at 0, 24, and 48 h during imbibition, and four replications for each treatment at each sampling time were used. Different small letters on top of the bars indicate significant differences (LSD, $\alpha = 0.05$) among treatments at the same imbibition time. Error bars indicate ± SE of mean ($n = 4$). For additional explanations, please see Figure 1.
Arabidopsis seeds have evolved a mechanism to regulate the abundance of the antagonistically acting hormones ABA and GA before germination. GA increased synthesis arising from diminished ABA by downregulating AtNCED9 and upregulating AtCYP707A1 and AtCYP707A2 (Topham et al., 2017). The mechanism also appeared in this study, and H₂O₂ and GA significantly reduced the ABA content at 24 h of imbibition and obviously increased the endogenous GA content at 0 and 24 h, and kept a lower level of the ABA/GA ratio during imbibition (Supplementary Figure S3). Exogenous GA reduced the expression of the ABA biosynthesis genes: NtNCED1 from 0 to 48 h of imbibition, and NtNCED3 (Figure 5) and NtABI5 at 24 h (Figure 7). H₂O₂ mainly downregulated the transcription level of NtNCED3 (Figure 5). Concomitantly, at 0 h of imbibition, ABA metabolic genes were highly upregulated by exogenous GA (12~17-fold of H₂O treatment), followed by H₂O₂ (9~14-fold of H₂O treatment) (Figure 6). However, DPI did not affect the expression of NtCYP707A1 and NtCYP707A2. This result may hint that H₂O₂ affected NtCPY707A through GA rather than directly affecting them. Plenty of studies have shown that H₂O₂ affects GA content by upregulating synthetic gene and downregulating catabolism gene during seed germination (Liu et al., 2010; Gomes and Garcia, 2013). Further evidence has shown that H₂O₂ markedly upregulated NtGA20OX2 (50-fold of H₂O at 0 h) and NtGA3OX2 (10-fold of H₂O at 0 h) (Figure 8) from 0 to 48 h during imbibition, and downregulated NtGA20OX1 (Figure 9). Notably, exogenous GA enhanced the production of endogenous GA by upregulating NtGA20OX2 (Figure 8). This
seems to be a self-induced feedback regulation. On the whole, these results suggest that there is a crosstalk between GA and \( \text{H}_2\text{O}_2 \) in mediating the homeostasis of ABA and GA.

On the other hand, the expression of GA signaling transduction genes \( \text{NtGID1} \) and \( \text{NtGID2} \) increased and the level of the seed germination inhibitor gene \( \text{NtRGL2} \) decreased after seed priming by GA or \( \text{H}_2\text{O}_2 \) (Figure 10), as different as possible from Uni or DPI. Cellular GA binds to the receptor GID, and the GID–GA complex interacts with DELLa to trigger the ubiquitination and destruction of RGL2, and induce seed germination (Arizumi and Steber, 2007; Golldack et al., 2013). GA or \( \text{H}_2\text{O}_2 \) downregulated the expression of \( \text{NtABI3} \) and \( \text{NtABI5} \), while Uni or DPI upregulated their expression. ABI3 and ABI5 were considered as the final downstream repressor of seed germination in the counterbalance of ABA and GA signals (Carles et al., 2002; Piskurewicz et al., 2008), and this was consistent with the RGL2-ABI5 model module that integrated the GA and ABA signaling pathways during seed germination (Liu et al., 2016). Another study has shown that \( \text{H}_2\text{O}_2 \) affects the ABA signal by inactivating ABI1 and ABI2 (Neill S. et al., 2002). These results suggest that there is a crosstalk between GA and \( \text{H}_2\text{O}_2 \) in mediating the signal transduction of ABA and GA.

Seed germination is a complex process controlled by many mechanisms, including the roles of GA and ABA (Toh et al., 2008), as well as radicle emergence (Guo et al., 2013), endosperm weakening (Zhang et al., 2014), and mobilization of storage reserves (Kelly et al., 2011; Han et al., 2013). During seed germination, lipolysis was followed by the gluconeogenesis pathway mainly conducting glucose production and sucrose used for seedling growth (Eckardt, 2005; Yan et al., 2014). For oilseeds, effective degradation of storage lipids is crucial for supporting respiratory substrates and energy generation, leading to successful seed germination and seedling establishment (Graham, 2008). Tobacco seeds contained higher lipid (30%) and lower sugar (10%) contents compared to the contents before priming (data are not shown), but the highest contents of soluble sugar in tobacco seeds was recorded at the end of priming (Supplementary Figure S3, 0 h). In addition, the change pattern of the ICL activity, which was involved in gluconeogenesis from lipid metabolism, was correlated with soluble sugar content during imbibition (Figure 3). Previous research conducted on oilseed species Arabidopsis (Meyer et al., 2012), sunflower (Raineri et al., 2016), and Brassica rapa (Gu et al., 2016) had also indicated that the levels of triacylglycerols, as components of lipids, decreased gradually during germination, suggesting that oilseed germination depends on oil breakdown and gluconeogenesis (Pritchard et al., 2002; Kelly et al., 2011).

Moreover, ABA and GAs have opposite roles in storage oil breakdown in the embryo (Penfield et al., 2006). Uni and DPI obviously increased the ABA content at 0 h of imbibition, and
decreased the ICL activity at 0 and 24 h, significantly in DPI. ABA content increase accompanied with the reduction of ICL activity in Uni and DPI, this result was in agreement with the studies showed that exogenous ABA inhibited the activity of ICL in castor bean endosperm and Brassica rapa (Marriott and Northcote, 1977; Finkelstein and Lynch, 2000), and GA was able to reverse the inhibitory effect of ABA. However, it was also interesting to note that GA significantly decreased the endogenous ABA content at 24 and 48 h (Figure 4) but was ultimately ineffective in increasing the ICL activity (Figure 3); on the contrary, the ICL activity induced by H$_2$O$_2$ significantly increased at 0 and 24 h of imbibition without a decline in the ABA content (Figure 3A). Remarkably, exogenous GA obviously upregulated the expression of NtRBOH$_H$, encoded the NADPH oxidase biosynthesis genes and was involved in the generation of H$_2$O$_2$, triggering the accumulation of H$_2$O$_2$ content at 0 and 24 h of imbibition in tobacco seeds (Figure 2). It could be hypothesized that both exogenous GA and H$_2$O$_2$ increased the endogenous H$_2$O$_2$ content to enhance the expression of NtICL (Figure 11) and promote ICL activity (Figure 3); only in the case of exogenous H$_2$O$_2$ priming, the expression of NtICL was reflected in a significant promotion of ICL activity. The ABA/GA ratio of Uni and DPI was obviously high at 0 and 24 h (Supplementary Figure S3), but the ICL activity of Uni and DPI was low at 0 and 24 h (Figure 3); the ABA/GA ratio of GA and H$_2$O$_2$ kept low levels at 0 and 24 h but the ICL activity of GA and H$_2$O$_2$ kept high levels at 0 and 24 h. The present results support the conclusion that not the contents of ABA and GA, but the low ABA/GA ratio and the high ROS contents were both responsible for enhancing ICL activity. Previous studies by Yanik and Donaldson (2005) showed that a very high concentration of H$_2$O$_2$ inactivates ICL and degrades its product, glyoxylate, when CAT is inactive in castor beans. Similar results were also confirmed by Jeon et al. (2011) who showed

![Figure 12](image-url)
that ICL activity was induced by a reasonable concentration \( \text{H}_2\text{O}_2 \) in \textit{Bradyrhizobium japonicum}, as was evident at both the transcriptional and translational levels. In addition, earlier results indicated that the amounts and activities of ROS scavenging enzymes were strongly downregulated in barley treated with GA, thus increasing the \( \text{H}_2\text{O}_2 \) concentration. Nevertheless, ABA had the opposite effects (Fath et al., 2001). Curiously, \( \text{H}_2\text{O}_2 \) treatment had a weak effect on the \( \text{NiRBOH} \) expression levels (\textbf{Figure 11A}). In \textit{N. benthamiana}, RBOH localized in the plasma membrane (PM) and the Golgi apparatus. PM-localized RBOH oxidase has been identified as a major source of ROS (Lherminier et al., 2009). ROS accumulation preceded RBOHD transcript- and protein-upregulation, indicating that ROS resulted from the activation of a PM-resident pool of enzymes, and that enzymes newly addressed to the PM were inactive (Noirot et al., 2014). Subcellular trafficking from Golgi to the PM was a potential determinant of RBOH activity, and this process regulation has mainly been addressed by considering the variation of gene expression or other signaling molecules, such as nitric oxide (NO) (Noirot et al., 2014). Both NO and ROS are known to react with each other and a balanced production between intracellular ROS and NO has been shown to be critical to the hypersensitive response (Delledonne et al., 2001). Yun et al. (2011) found that NO mediated S-nitrosylation of \( \text{AtRBOHD} \) governs a feedback regulation between ROS and NO; \( \text{H}_2\text{O}_2 \) suppresses \( \text{NiRBOH} \) expression through other signaling when \( \text{H}_2\text{O}_2 \) is sufficient in the cell. Signaling activities of RBOH-derived ROS are probably also modulated by other ROS sources including xanthine oxidase, amine oxidase, and a cell wall peroxidase. It is possible that different stimuli activate specific \( \text{H}_2\text{O}_2 \)-generating enzymes (Neill S.J. et al., 2002). Multiple source pathways of \( \text{H}_2\text{O}_2 \) may also be responsible for the inconsistency in \( \text{H}_2\text{O}_2 \) concentration and \( \text{NiRBOH} \) expression.

The present results show that the application of DPI and Uni increased ABA content and decreased GA content, by regulating the corresponding gene expression and, eventually, delayed the germination time and reduced the GP. The experiment revealed that \( \text{H}_2\text{O}_2 \) and GA were essential for tobacco seed germination mainly because of downregulating the ABA/GA ratio and inducing reserve composition mobilization to mutually promote seed germination. In addition, the potential role of ROS in seed germination was proposed that it affected the homeostasis and the signal transduction of GA and ABA by activating or inactivating gene expressions, eventually decreasing the ABA/GA ratio. ROS is also attributed to enhanced ICL activity at a higher concentration (\textbf{Figure 12}). However, in this work, the interaction effects between ROS, GA, and ABA were primary determined, and the underlined mechanisms need further study.

**AUTHOR CONTRIBUTIONS**

ZL conceived and designed the experiments. ZL, YG, YZ, CL, and DG performed the experiments. ZL and YG analyzed the data. All authors contributed reagents, materials, and analysis tools. ZL, YG, and YJG wrote the paper.

**FUNDING**

This research was supported by the National Natural Science Foundation of China (Nos. 31201279, 31371708, and 31671774), Zhejiang Provincial Natural Science Foundation (Nos. LY15C130002 and LZ14C130002), Science and Technology Project of Yunnan Provincial Tobacco Company (201835000241035), Dabeinong Funds for Discipline Development and Talent Training in Zhejiang University and Jiangsu Collaborative Innovation Center for Modern Crop Production, China.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01279/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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