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Engineering *Bacillus velezensis* with high production of acetoin primes strong induced systemic resistance in *Arabidopsis thaliana*

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ABSTRACT

Many plant growth promoting rhizobacteria such as *Bacillus velezensis* GJ11 can produce acetoin to trigger induced systemic resistance (ISR) in plants. For improving acetoin production, the mutant strains were respectively constructed by knockout of the gene of *bdh* (2,3-butanediol dehydrogenase) and *gdh* (glycerol dehydrogenase) in GJ11, but only GJ11Δ*bdh* produced a high level of acetoin triggering strong ISR against *Pseudomonas syringae* infection in plants. GJ11Δ*bdh* could induce H_2O_2 accumulation in plants by producing a high level of acetoin. H_2O_2 was necessary for triggering ISR against the pathogen infection because after scavenging H_2O_2 with ascorbic acid or catalase, the inhibition role to pathogen infection induced by acetoin almost disappeared in plants. Further investigation found the plants treated with GJ11Δ*bdh* in an obvious "priming" state, in which the mild immune response was observed such as a slight increase of H_2O_2 production, callose deposition, and enzymes activity related with defence response (*e.g.* POD, PAL and PPO). The plants in "priming" could rapidly respond to the pathogen infection accompanying with a significant increase of H_2O_2 production, callose deposition, and enzymes activity. Collectively, this study provides new insight into the role of acetoin as a strong elicitor of defense response, and ascribes a new approach to construct the mutant strains with high production of acetoin for triggering stronger ISR against pathogens infection in plants.

1. Introduction

Crops are constantly challenged by numerous plant diseases. For a sustainability and environmental safety of agricultural production, the eco-friendly approaches are investigated like utilization of plant growth promoting rhizobacteria (PGPRs) that are a group of root-colonizing bacteria in the rhizosphere of many plants, for bio-control of plant diseases (Fincheira et al., 2016; Gopalakrishnan et al., 2015; Pérez-Flores et al., 2017). Until now, many PGPRs have been reported to colonize root systems against foliar diseases by establishment of beneficial interactions with plants, including *Pseudomonas fluorescens*, *P. putida*, *Bacillus pumilus*, *B. amyloliquefaciens*, *B. subtilis*, *B. velezensis*, and so on (Kierul et al., 2015; Pérez-Flores et al., 2017; Ryu et al., 2004). These interactions contain triggering resistance against pathogens. This mechanism of induction of systemic resistance by root colonizing rhizobacteria in aerial plant parts is referred to as induced systemic resistance (ISR). PGPRs-elicited ISR has been observed in many crops accompanying with a reduced susceptibility to phytopathogens.

Recently, researchers found that PGPRs can produce volatile organic compounds (VOCs) to induce ISR against various plant pathogens and promote plant growth (Gopalakrishnan et al., 2015; Fincheira et al., 2016; Lee et al., 2012; Pérez-Flores et al., 2017). In particular, one well-characterized volatile is 2,3-butanediol (2,3-BD) and its precursor 3-hydroxy-2-butanone (acetoin), which are found in multiple bacterial species such as *Bacillus* spp., *Aerobacter* spp., *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., *etc* (Effantin et al., 2011; Han et al., 2006; Ryu et al., 2003, 2004; Yi et al., 2016). Since the original demonstration that exogenous application of 2,3-BD or the exposure to 2,3-BD-producing strains (*e.g. B. subtilis*) triggers resistance against the bacterial pathogen *Erwinia carotovora* in *Arabidopsis thaliana* (Ryu et al., 2004), several studies have demonstrated a positive effect of this volatile compound on plant resistance. For example, application of 2,3-BD to tobacco could increase the resistance against *Colletotrichum orbiculare* (D'Alessandro et al., 2014). Different from 2,3-BD, there are rare reports that acetoin, the precursor of 2,3-BD, is used to trigger ISR against pathogens in plants (Rudrappa et al., 2010).

Many *Bacillus* spp. such as *B. amyloliquefaciens, B. subtilis*, *B. methylotrophicus*, *B. velezensis* and others, can produce both of 2,3-BD and acetoin in the presence of glucose as carbon source (Fincheira et al., 2016; Gotor-Vila et al., 2017; Han et al., 2006; Pérez-Flores et al., 2017;

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Ryu et al., 2004, 2003). *Bacillus* synthesize 2,3-BD from pyruvate by a condensation and oxidation step. Firstly, pyruvate is converted to acetolactate by acetolactate synthase (AlsS) under oxygen-limiting conditions. Subsequently, acetolactate is decarboxylated to acetoin by acetolactate decarboxylase (AlsD) (Chung et al., 2016; Kierul et al., 2015). Acetoin is the last synthesis intermediate for 2,3-BD, and metabolic conversion of acetoin to 2,3-BD is reversible in *Bacillus* (Effantin et al., 2011). In *B. subtilis* acetoin is reduced to 2,3-BD in the catalysis of 2,3-BD dehydrogenase (BDH) (D'Alessandro et al., 2014). Differently, the enzymes are *meso*-2,3-BD dehydrogenase (BudC) that catalyzes the conversion between acetoin and *meso*-2,3-BD (Qi et al., 2014), and glycerol dehydrogenase (GDH) that catalyzes the conversion between acetoin and *D*-2,3-BD (Qiu et al., 2016) in *B. licheniformis.* Thereby, different *Bacillus* strains produce different racemic mixture of 2,3-BD such as *D*-, *L*- and *meso*- 2,3-BD (Han et al., 2006). However, it is still unknown which enzyme catalyzes the conversion between acetoin and 2,3-BD in *B. velezensis*.

Many PGPRs including *Bacillus* species (*e.g. B. velezensis*) can reside and actively colonize the living tissue of host plants without substantively harming them hence also called as endophytic bacteria (Taghavi et al., 2015). Researchers have reported that *B. velezensis* has potential to control several plant diseases such as *Fusarium* wilt in strawberries, wheat powdery mildew, *Fusarium* head blight, and so on by producing volatile compounds (Huang et al., 2017; Lim et al., 2017). Previously, we isolated an endophytic bacterium of *B. velezensis* GJ11 from tobacco rhizospheres with an activity against phytopathogenic fungi. In this study, we found GJ11 could produce acetoin to induce ISR in plants. For higher production of acetoin, we constructed several mutant strains based on GJ11: 1) blocked the metabolic pathway from acetolactate to acetoin and 2,3-BD by knockout of *alsD* (Chung et al., 2016; Kierul et al., 2015), and 2) blocked the metabolic pathway from acetoin to 2,3-BD by knockout of *bdh* (D'Alessandro et al., 2014) or *gdh* (Qiu et al., 2016) in GJ11. Compared to GJ11, the *alsD* null mutant strain could not produce both acetoin and 2,3-BD, the *bdh* null mutant strain could produce more acetoin, and the *gdh* null mutant strain could produce more 2,3-BD in broth. Among them, the *bdh* null mutant strain with higher production of acetoin triggered a stronger ISR against the pathogen of *Pseudomonas syringae* pv. *tomato* DC3000 (termed as *Pst* DC3000) than the wild-type strain. Further studies revealed that acetoin could induce H_2O_2 production to enhance the activity of defense-related enzymes including peroxidase (POD), polyphenoloxidase (PPO) and phenylalnine ammonialyase (PAL) but not catalase (CAT), and callose deposition in plants. Although *B. velezensis* has been reported to trigger ISR by producing acetoin (Rudrappa et al., 2010), the production of acetoin is generally low in the wild type strain that does not reach the functional levels required for the beneficial action in plants (Chung et al., 2016). Here, our results highlighted a way to use genetic engineering *B. velezensis* strains with high production of acetoin for triggering a stronger ISR against plant diseases.

2. Materials and methods

2.1. Strains, plasmids, primers and reagents

The bacterial strains, plasmids and primers used in this study were listed in Tables 1 and 2, respectively. *Bacillus velezensis* GJ11 was previously isolated from the *rhizospheric* soil of tobacco from XuanEn county, Hubei Province, China, with an activity against the phytopathogenic fungus of *Phytophthora nicotianae*.

The materials for DNA manipulation including T4 DNA ligase, DNA marker, *Pfu* DNA Polymerase and Plasmid Miniprep Kit were from Takara Bio (China). Nucleotide sequences were determined by Sangon Biotech, Shanghai, China. Chemically synthesized acetoin was purchased from Biosharp, China. All other chemicals were of analytical grade supplied by Sinopharm Chemical Reagent (China).

2.2. Construction of knockout strains

The genes including *bdh*, *gdh* and *alsD* were deleted by the double crossover homologous recombination method (Qi et al., 2014). The genomic DNA was extracted from *B. velezensis* GJ11. Firstly, two approximately 500 bp arms homologous to the 5' and 3' coding regions of the above genes were amplified by PCR from the genomic DNA of *B. velezensis* GJ11 by primers LF & LR for L arm, and RF & RR for R arm (Table 2), then ligated by splicing with Overlapping Extension PCR (SOE-PCR) using primers LF and RR, respectively. After digestion by *BamH* I and *Xba* I, the DNA fragments were subcloned into the vector T2(2)-ori with a temperature-sensitive replicon from *B. subtilis* to promote the single crossover (Qi et al., 2014).

The resulting plasmids were used for transformation of *B. velezensis* GJ11 by using the method described by Qi et al. (2014). Transformants were selected by Kanamycin resistance (20 μg/mL) followed with verification by PCR using the related LF and RR primers (Table 2). The selected transformants were cultured in Luria-Bertani (LB) medium containing Kanamycin at 45 °C for 8 h to promote the first crossover, then the mutants were selected by PCR with the related single-crossover dan-LF & dan-RR primers (Table 2). The selected colonies with single crossover were picked up and cultured in LB medium at 37 °C for 8 h, and this process was repeated for 6 times. After serial transfer without antibiotics, the cells were spread on LB agar plates, then replicated on Kanamycin plates for selecting sensitive colonies. The Kanamycin - sensitive colonies might include the ones with second crossover, which were further confirmed by PCR with the related LF & RR primers listed in Table 2, respectively.

2.3. Analysis of acetoin and 2,3-BD in broth

For fermentation, the wild-type strain GJ11 and mutant strains of GJ11Δ*bdh*, GJ11Δ*gdh*, and GJ11Δ*alsD* were inoculated into 30 mL LB medium in 250-mL flasks, then incubated at 37 °C and 180 r/min for 11 h until the OD_{600} of culture reached to ˜ 4.2. The culture was transferred into 50 mL fresh LB medium containing 70 g/L glucose in 250-mL flasks, and cultivated at 37 °C and 180 r/min, respectively. The samples were collected at desired intervals to determine the content of acetoin and 2,3-BD with gas chromatography (GC) by our previous methods (Qi et al., 2014).

2.4. Analysis of hypersensitive reaction induced by bacterial strains

GJ11 and mutant strains were cultured at 37 °C and 180 r/min for 48 h, then the broth supernatant and bacterial cells were collected by centrifugation of 10 mL culture. The cell pellets were resuspended with 10 mL distilled water. *A. thaliana* was used for detecting the activity of acetoin and bacterial strains to induce hypersensitive response (HR) in plants. The leaves of *A. thaliana* with six-week old (6 seedlings per group) were injected with 20 μL acetoin (10 g/L), bacterial broth, bacterial broth supernatant and bacterial cells of GJ11 and mutant strains by 1 mL syringe without needle. After 24 h, the leaves were collected and stained with trypan blue (40 mg trypan blue, 10 mL lactic acid, 10 mL phenol and 10 mL glycerol dissolved in 10 mL distilled water), treated with ethanol, and observed by microscope.

2.5. Detecting ISR towards Pst DC3000 elicited by bacterial strains

GJ11 and mutant strains were fermented in LB medium with 70 g/L glucose at 37 °C for 48 h, then the broth supernatants were collected by centrifugation for irrigating *A. thaliana* seedlings (6-week old, 10 seedlings per group) at 5 mL per seedling. Five mL acetoin (10 g/L) was also used for irrigating *A. thaliana* seedlings. Three days latter, the seedlings were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 (termed as *Pst* DC3000, 1×10^8 CFU mL^{-1}) by spraying leaves, or by injecting leaves with 20 μL of *Pst* DC3000 (2.5×10^6 CFU

Table 1
Bacterial strains and plasmids used in this study.

Bacterial Strains/ plasmids	Properties	Source
Escherichia coli DH5α	Used for plasmid amplification	Stored in this lab
Bacillus velezensis GJ11	Wild-type with production of *D*-2,3-BD and acetoin	Stored in this lab
T2(2)-ori	$\text{ori}_{\text{pUC}}/\text{ori}_{\text{ts}}$, temperature-sensitive, Kan^{r}	Stored in this lab
T2-Δ*bdh*	Plasmid based on T2(2)-ori for knock-out of *bdh*	This study
T2-Δ*gdh*	Plasmid based on T2(2)-ori for knock-out of *gdh*	This study
T2-Δ*alsD*	Plasmid based on T2(2)-ori for knock-out of *alsD*	This study
GJ11Δ*bdh*	*bdh* knock-out strain	This study
GJ11Δ*gdh*	*gdh* knock-out strain	This study
GJ11Δ*alsD*	*alsD* knock-out strain	This study
Pseudomonas syringae pv. tomato DC3000	Pathogenic bacterium with Kanamycin resistance	Stored in this lab

Kan^{r}: Kanamycin resistance; ori_{ts}: thermosensitive replicon. "Δ" means the deleted gene. *bdh*, *gdh*, and *alsD* is the gene of 2,3-butanediol dehydrogenase, glycerol dehydrogenase, and α-acetolactate decarboxylase, respectively.

mL^{-1}) after being mixed with 40 μmol/L ascorbic acid (ASC) or 5000 U/mL catalase by 1 mL syringe without needle. The seedlings only injected with 20 μL of *Pst* DC3000 on leaves were used as control.

After 3 days, the leaves were collected and sterilized by using 70% (v/v) ethanol for 40 s, then washed with sterilized water 3 times, and homogenized in 2 mL 0.85% (w/v) NaCl solution. After serial dilutions, 100 μL suspension was spread on KB plate (tryptone 20 g, K_2HPO_4 1.5 g, $MgSO_4·7H_2O$ 1.5 g, glycerol 10 mL, and agar 15 g dissolved in 1 L water, pH 7.2) with 50 μg/mL Kanamycin, cultured at 28 °C for 48 h, and the bacterial colony was counted for calculating the *Pst* DC3000 content per gram of fresh leaf (CFU g^{-1}).

2.6. Detecting cellular defensive responses elicited by bacterial strains

A. thaliana seedlings (six-week old, 10 seedlings per group) were irrigated by 5 mL acetoin (10 g/L), or the broth supernatant of GJ11, GJ11Δ*bdh* and GJ11Δ*alsD*, then inoculated with *Pst* DC3000 by spraying leaves as described above. After that, the leaves were collected and stained with DAB to detect the accumulation of H_2O_2, a kind of reactive oxygen species (ROS), in plants. Briefly, the leaves were stained by DAB Kit (Beyotime Biotechnology, China) with shaking at 100 rpm for 4 h under dark, then treated with ethanol at 90 °C for 15 min, and observed by microscope.

H_2O_2 in leaves was also quantitatively determined (Choi and Hwang, 2007). Fe^{2+} can be oxidized to Fe^{3+} by H_2O_2, which is able to react with xylenol orange to form the purple complex compounds with a maximum absorption at 560 nm. Briefly, the leaves of *A. thaliana* seedlings were homogenized in 1 mL water, centrifuged at 12,000 rpm for 1 min, then 300 μL of supernatant was collected to mix with 2 mL xylenol orange solution (This was prepared by mixing Solution I with Solution II at a ratio of 1:100 (v/v), in which Solution I contains 25 mmol/L $FeSO_4$, 25 mmol/L $(NH_4)_2SO_4$ and 2.5 mol/L H_2SO_4, and Solution II contains 125 μmol/L xylenol orange and 100 mmol/L sorbitol). The mixture was incubated at room temperature for 30 min, then the OD_{560} value was detected by spectrophotometer to calculate the H_2O_2 content in leaves.

Callose in leaves was detected as described by the literature (Luna et al., 2011). Callose can be stained by aniline blue showing green fluorescence. Briefly, the leaves of *A. thaliana* were fixed with 1% (v/v) glutaraldehyde solution overnight, treated with ethanol at 90 °C for 15 min, and stained with 0.1% (w/v) aniline blue for 12 h, then observed by fluorescence microscope after being extensively washed by 50% (v/v) ethanol solution.

2.7. Detecting activity of plant defense related enzymes induced by acetoin

A. thaliana seedlings (6-week old, 10 seedlings per group) were irrigated by 5 mL acetoin (10 g/L) per seedling, then sprayed with *Pst* DC3000 as above. The leaves of *A. thaliana* were collected at 0, 12, 24 and 48 h after irrigating with acetoin, and at 0, 6, 12, 24, 30, 36 and 48 h after inoculation with *Pst* DC3000. The collected leaves were used for detecting the activity of plant defense related enzymes including POD, PAL, PPO and CAT, respectively.

POD activity was detected as described by the previous literature

Table 2
Primers for PCR used in this study.

Name	Primer sequence(5'-3')	Purpose	Tm/°C
bdh-LF	CGGGATCCAATTATACACCAAGAAG	Amplification of L arm of *bdh*	51
bdh-LR	TGATATTCACCATTTGTTCATCAAG		
bdh-RF	AACAAATGGTGAATATCAGACACAT	Amplification of R arm of *bdh*	57
bdh-RR	GCTCTAGATTGACGGTGTTGATGTT		
bdh-dan-LF	AGCACTTGCATCAGAAGAGT	Verification of single crossover of *bdh*	53
bdh-dan-RR	AATGCTGTCCAGTTCCTTAG		
gdh-LF	CGGGATCCCGTACCGAGTATCGTGTATA	Amplification of L arm of *gdh*	51
gdh-LR	CCACGTCATCAGATGTCCGCCGAT		
gdh-RF	GGACATCTGATGACGTGGCAGATG	Amplification of R arm of *gdh*	51
gdh-RR	GCTCTAGAAGCGGTGAACCCTTGAAGAA		
gdh-dan-LF	TAGATCCGTCAGCGATAAAG	Verification of single crossover of *gdh*	53
gdh-dan-RR	AATCGTATGCCATGATGCAA		
alsD-LF	CGGGATCCATGCAATTGATGACGAC	Amplification of L arm of *alsD*	44
alsD-LR	GCTTCCTTCTGTTGATACTTGATAG		
alsD-RF	GTATCAACAGAAGGAAGCCCGGAAT	Amplification of R arm of *alsD*	46
alsD-RR	GCTCTAGAATTGTCGGATGAAGATG		
alsD-dan-LF	GCAGCCGTGA ACAAGAAGTG	Verification of single crossover of *alsD*	53
alsD-dan-RR	AACGGGATATATCAGCACAA		

"GGATCC" represents *BamH* I cleavage site sequence, "TCTAGA" represents *Xba* I cleavage site sequence, and the letters with dashed line represent the SOE overlap sequence.

(Soffan et al., 2014). Briefly, 0.25 g leaves of *A. thaliana* were homogenized in 3 mL pre-cooled phosphate buffered saline solution (PBS) containing 0.1 g polyvinylpyrrolidone (PVP), then the supernatant was collected by centrifugation at 12,000 rpm and 4 °C for 10 min. One mL of supernatant was mixed with 1 mL of 0.05 mol/L PBS and 1 mL of 1% (w/v) guaiacol solution, incubated at 30 °C for 5 min, then added with 1 mL of 0.3% (v/v) H_2O_2. After that, the OD_{470} value was measured by spectrophotometer at the start and end of reaction. Under these conditions, 1 U of POD activity was defined as the variance of 0.01 of OD_{470} value per minute, and POD activity was expressed as change of absorbance/min/g fresh leaves.

The activity of PAL, PPO and CAT was determined as described by the previous literature, respectively (Song and Wang, 2011). Briefly, the leaves were homogenized in 3 mL of pre-cooled boracic acid buffer (0.05 mol/L, pH 8.4), then the supernatant was collected by centrifugation at 12,000 rpm and 4 °C for 20 min. For analysis of PAL activity, 0.2 mL of supernatant was mixed with 3.8 mL of boracic acid buffer (0.1 mol/L) and 1 mL of L-Phenylalanine solution (0.02 mol/L), incubated at 40 °C for 60 min, then added with 1 mL HCl solution (1 mol/L) to terminate the reaction. First reading was done before incubation by a spectrophotometer at 290 nm and at room temperature to get an initial absorbance. Second reading was done after 1 h incubation at 40 °C. Under these conditions, 1 U of PAL activity was defined as the variance of 0.01 of OD_{290} value per minute, and PAL activity per leaf gram were expressed as change of absorbance at 290 nm (A_{290}/g of leaf) from the first and second reading.

For analysis of PPO activity, 0.1 mL of supernatant was mixed with 1.5 mL of PBS (0.05 mol/L, pH 7.0) and 1.5 mL of catechol solution (0.02 mol/L). Catechol was used as a substrate for the enzyme. First reading was done before incubation by a spectrophotometer at 420 nm and at room temperature to get an initial absorbance. Second reading was done after 1 h incubation at 38 °C. Under these conditions, 1 U of PPO activity was defined as the variance of 0.01 of OD_{420} value per minute, and PPO activity units per leaf gram were expressed as change of absorbance at 420 nm (A_{420}/g of leaf) from the first and second reading.

For analysis of CAT activity, 0.2 mL of supernatant was mixed with 1.5 mL of PBS (0.05 mol/L, pH 7.0) and 0.3 mL of H_2O_2 solution (0.1 mol/L), then the OD_{240} value was continuously determined by spectrophotometer for 4 min. Under these conditions, 1 U of CAT activity was defined as the decrease of 0.1 of OD_{240} value per minute, and CAT activity units per leaf gram were expressed as change of absorbance at 240 nm (A_{240}/g of leaf) from the start to end reading.

2.8. Colonization of bacterial strains in rhizospheric soil and plant root

GJ11 and GJ11Δ*bdh* were transformed by T2(2)-ori plasmid with Kanamycin resistance. The positive transformants were selected by Kanamycin resistance, then cultured at 37 °C and 180 rpm for 24 h. The culture was centrifugated at 4 °C and 8000 rpm for 10 min, and the pellets were collected and resuspended in water at 5×10^9 CFU/mL. The cells suspension was used for irrigating *A. thaliana* seedlings (six-week old, 6 seedlings per group) at 5 mLper seedling. After 7, 14, 21, 28 and 42 days, the rhizospheric soil and root of seedlings were collected for counting GJ11 and GJ11Δ*bdh*, respectively. Briefly, 1 g of rhizospheric soils were suspended in 19 mL sterilized water, then used for spreading plates with Kanamycin (20 μg/mL) after serial dilutions. One hundred mg of roots were sterilized by 70% ethanol for 40 s, washed by sterilized water 3 times, then homogenized in sterilized water for spreading LB agar plates with Kanamycin (20 μg/mL).

2.9. Statistical analysis of data

All experiments were repeated in triplicates. Data from different groups were compared by a one-way analysis of variance (ANOVA).

3. Results

3.1. Mutant strains with modified pathway for synthesis of acetoin and 2,3-BD

The constructed plasmids for knockout of *bdh*, *gdh* and *alsD* gene (Table 1) were used for transformation of *B. velezensis* GJ11, then the positive strains were selected by Kanamycin-resistance, and further verified by PCR and sequencing of PCR products. A DNA fragment about 2.2 kb was amplified from the genomic DNA of GJ11 as negative control, and the PCR product amplified from the genomic DNA of *bdh*, *gdh* and *alsD* knockout strain was about 1.4 (Fig. S1a), 1.4 (Fig. S1b) and 1.6 kb (Fig. S1c), indicating that these genes have been deleted in GJ11. Further analysis by nucleotide sequencing of the PCR products showed no mutation was found except for the *bdh*, *gdh* and *alsD* deletion, suggesting the null mutant strains of GJ11Δ*bdh*, GJ11Δ*gdh* and GJ11Δ*alsD* were successfully constructed.

3.2. Acetoin and 2,3-BD production of mutant strains

Knockout of *bdh*, *gdh* or *alsD* did not obviously influence the bacterial growth in LB medium (data not shown here). The production of acetoin and 2,3-BD of mutant strains were determined by GC. GJ11 could produce both of acetoin and 2,3-BD (mainly *D*-2,3-BD) in the broth (Fig. 1a). GJ11Δ*bdh* showed a high production of acetoin, which was significantly ($p < 0.01$) higher than the wild-type strain GJ11 (Fig. 1a&b). After fermentation for 96 h, GJ11Δ*bdh* achieved the highest acetoin production of 17.18 g/L in broth, which was about 2 times of GJ11 (Fig. 1b). On the other hand, the *D*-2,3-BD production of GJ11Δ*bdh* was significantly ($p < 0.01$) lower than the wild-type strain GJ11 (Fig. 1a&c).

Knockout of *gdh* gene could not improve the acetoin production (Fig. 1a&b), but could enhance the *D*-2,3-BD production that was significantly higher than GJ11 at several time points such as 48 and 120 h (Fig. 1a&c). Knockout of *alsD* blocked the carbon overflow metabolism to produce acetoin and 2,3-BD in *B. velezensis* (Fig. 1a). The production of acetoin and *D*-2,3-BD were both significantly reduced in the strain GJ11Δ*alsD* when compared to GJ11 (Fig. 1b&c).

3.3. Hypersensitive response induced by bacterial strains

GJ11 and mutant strains were determined for the activity to induce hypersensitive response (HR) that can be stained by trypan blue in plant leaves. Treatment with water or medium could not induce obvious HR in leaves, but treatment with acetoin could induce strong HR in *A. thaliana* (Fig. 2a). In addition to acetoin, treatment with the broth of GJ11, GJ11Δ*bdh* or GJ11Δ*gdh* could also induce HR in *A. thaliana* leaves. After being cultured for 72 h, the acetoin production was 6.1, 14.9, 6.8 g/L and 0.2 g/L for G11, GJ11Δ*bdh*, GJ11Δ*gdh* and GJ11Δ*alsD*, respectively. The broth of GJ11Δ*gdh* with high production of *D*-2,3-BD showed a similar HR to GJ11, while the broth of GJ11Δ*bdh* with high production of acetoin induced a much stronger HR than GJ11 in plant leaves (Fig. 2b). Different from GJ11Δ*bdh* and GJ11Δ*gdh*, the broth of GJ11Δ*alsD* without production of acetoin and *D*-2,3-BD could not induce obvious HR in leaves (Fig. 2b). The broth supernatant of GJ11 and GJ11Δ*bdh* could also induce obvious HR in leaves. Compared to GJ11, the broth supernatant of GJ11Δ*bdh* induced a stronger HR in plant leaves, but the broth supernatant of GJ11Δ*gdh* showed a weaker activity to induce HR than GJ11 (Fig. 2c). The broth supernatant of GJ11Δ*alsD* could not induce obvious HR in plant leaves when compared to GJ11 (Fig. 2c). Comparison with the broth supernatant, the bacterial cells only induced a weak HR in plants leaves (Fig. 2d), indicating the activity of bacterial broth to induce HR is mainly attributed to the supernatant of broth rather than the bacterial cells.

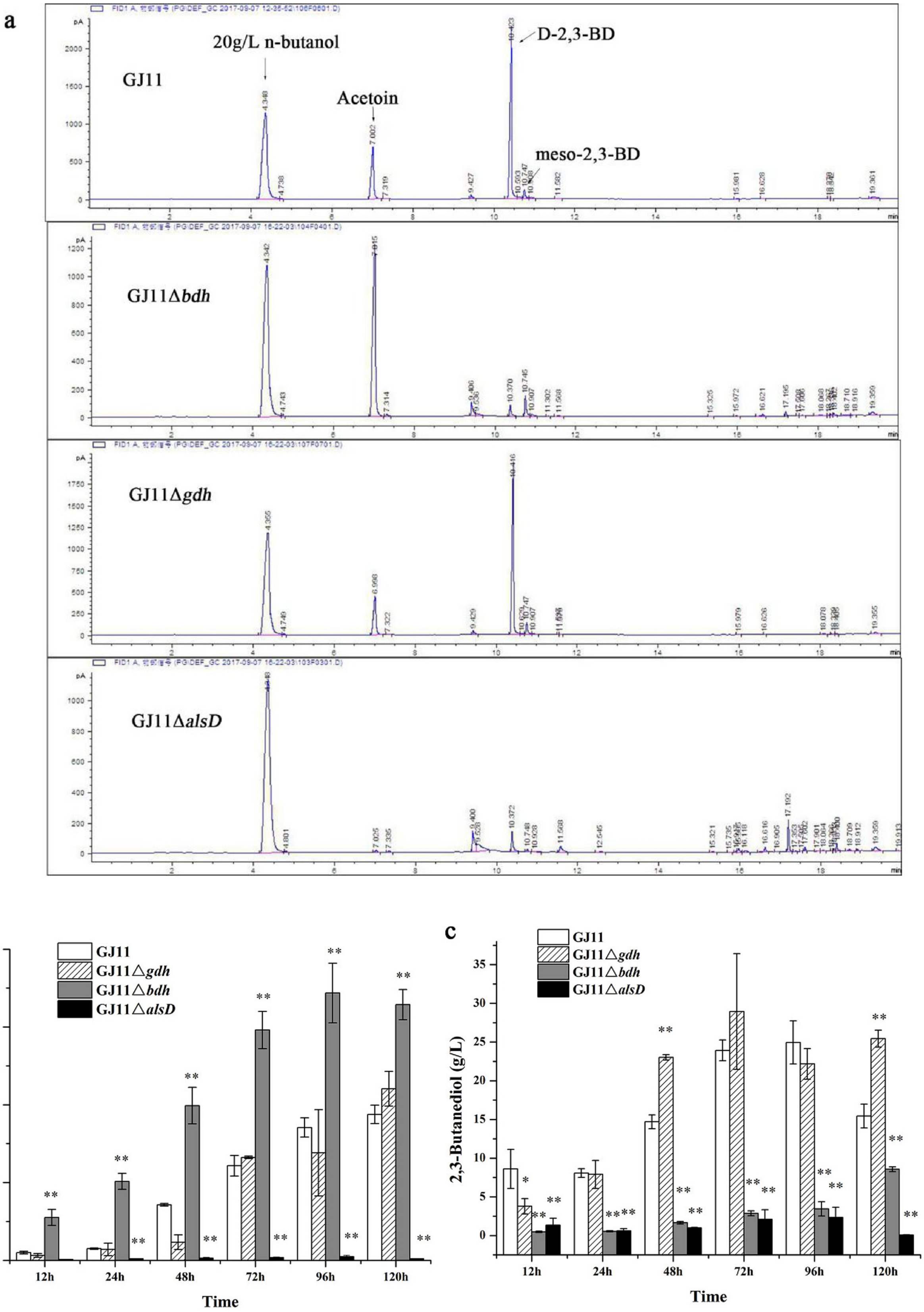

Fig. 1. Production of acetoin and 2,3-BD by GJ11 and mutant strains**. (a):** analysis of acetoin and 2,3-BD in the broth of bacterial strains by GC; **(b):** acetoin production at different time points of fermentation; **(c):** *D*-2,3-BD production at different time points of fermentation. ** and * represent very significant difference ($P < 0.01$) and significant difference ($P < 0.05$) compared to the wild-type strain, respectively. This experiment was repeated in triplicates, and N = 5 per group.

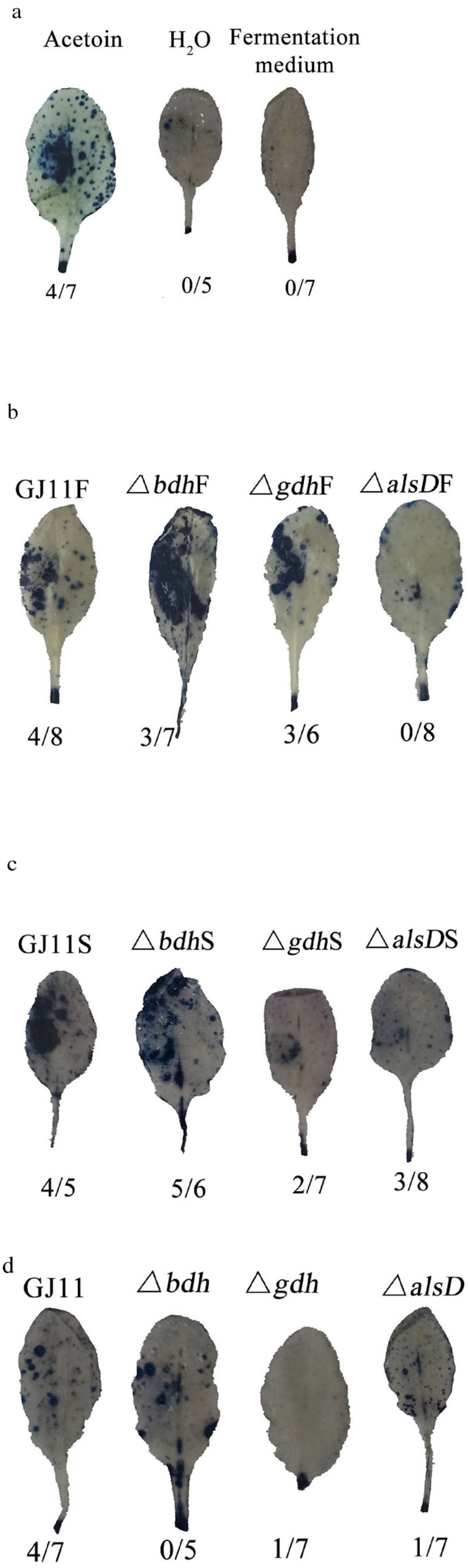

Fig. 2. Hypersensitive response (HR) induced by bacterial strains and acetoin in *A. thaliana*. (a): HR in plants induced by acetoin. **(b):** HR in plants induced by the bacterial broth. GJ11 (Δ*bdh*, Δ*gdh*, Δ*alsD*) F: Broth of GJ11 (GJ11Δ*bdh*, GJ11Δ*gdh*, GJ11Δ*alsD*). **(c):** HR in plants induced by the supernatant of bacterial broth. GJ11 (Δ*bdh*, Δ*gdh*, Δ*alsD*) S: Broth supernatant of GJ11 (GJ11Δ*bdh*, GJ11Δ*gdh*, GJ11Δ*alsD*). **(d):** HR in plants induced by the bacterial cells. GJ11 (Δ*bdh*, Δ*gdh*, Δ*alsD*): Bacterial cells of GJ11 (GJ11Δ*bdh*, GJ11Δ*gdh*, GJ11Δ*alsD*) suspended in water. Number below leaves means the number of leaves with HR / the number of total leaves treated. N = 6 per group, and this experiment was repeated in triplicates.

3.4. Bacterial strains with production of acetoin triggering ISR against Pst DC3000 in A. thaliana

In addition to HR, the strains with production of acetoin could also trigger ISR in *A. thaliana*. Seedlings were treated with acetoin or the bacterial broth by irrigation, then inoculated with *Pst* DC3000 *via* spraying leaves. Three days post inoculation, the plants in control group (treatment with water) showed serious symptom with large of yellow leaves. Compared to control, the plants treated with acetoin showed slighter symptom with few yellow leaves. Therefore, acetoin could reduce the disease severity induced by *Pst* DC3000 (Fig. 3a). Further investigation found *Pst* DC3000 (CFUs per gram fresh weight of the inoculated leaves) in the case of acetoin-treated plants was significantly lower than the control. The results indicated that acetoin can effectively trigger ISR protecting plants from pathogens infection.

We detected the dose-effect of acetoin on triggering ISR, and found acetoin could not effectively trigger ISR to inhibit the pathogen infection at a concentration less than 5 g/L. When the acetoin concentration increased to 10 g/L, ISR was obviously triggered to inhibit the pathogen infection in plants (Fig. 3b). 20 g/L of acetoin could also induce obvious ISR in plants that was similar to the dose of 10 g/L. Thereby, 10 g/L acetoin was selected for irrigating plants here. Results presented here indicated that acetoin has the potential to trigger ISR against *Pst* DC3000 pathogenesis in plants.

The bacterial strains with production of acetoin were also detected for the ability to trigger ISR in plants. *A. thaliana* leaves pre-treated with the broth supernatant of GJ11Δ*bdh* with high production of acetoin showed slight symptom without obvious yellow leaves observed, while the ones treated with the broth supernatant of GJ11Δ*alsD* without production of acetoin and *D*-2,3-BD showed an obvious symptom similar to the control (water) after inoculation by *Pst* DC3000 (Fig. 3c). Pre-treatment with the broth supernatant of GJ11Δ*gdh* wit high production of *D*-2,3-BD could not induce ISR to inhibit *Pst* DC3000 infection when compared to the control (water). It showed an obvious symptom similar to the control after inoculation by *Pst* DC3000 (Fig. 3c). Further investigation found the CFU pathogen number was significantly lower in the GJ11Δ*bdh* group compared to the control. However, the CFU pathogen number in GJ11Δ*alsD* or GJ11Δ*gdh* was similar to the control, which were both significantly higher than the GJ11Δ*bdh* group (Fig. 3c). Additionally, the disease incidence in plants pre-treated with the broth supernatant of GJ11Δ*bdh* was significantly lower than GJ11, GJ11Δ*alsD*, GJ11Δ*gdh* and control (H_2O) (Fig. 3c). The disease incidence was similar between GJ11Δ*alsD* and control (H_2O), but was significantly higher than GJ11, GJ11Δ*gdh* and GJ11Δ*bdh*. The disease incidence was similar among GJ11, GJ11Δ*gdh* and GJ11Δ*bdh* (Fig. 3c). These results indicated that pre-treatment with the broth supernatant of GJ11Δ*bdh* with high production of acetoin showed better control effects on *Pst* DC3000 infection than control and other treatments.

3.5. Defence-related responses in plants induced by acetoin and bacterial strains

PGPRs can trigger ISR to protect plants from pathogens infection by eliciting defence-related responses such as ROS (*e.g.* H_2O_2)

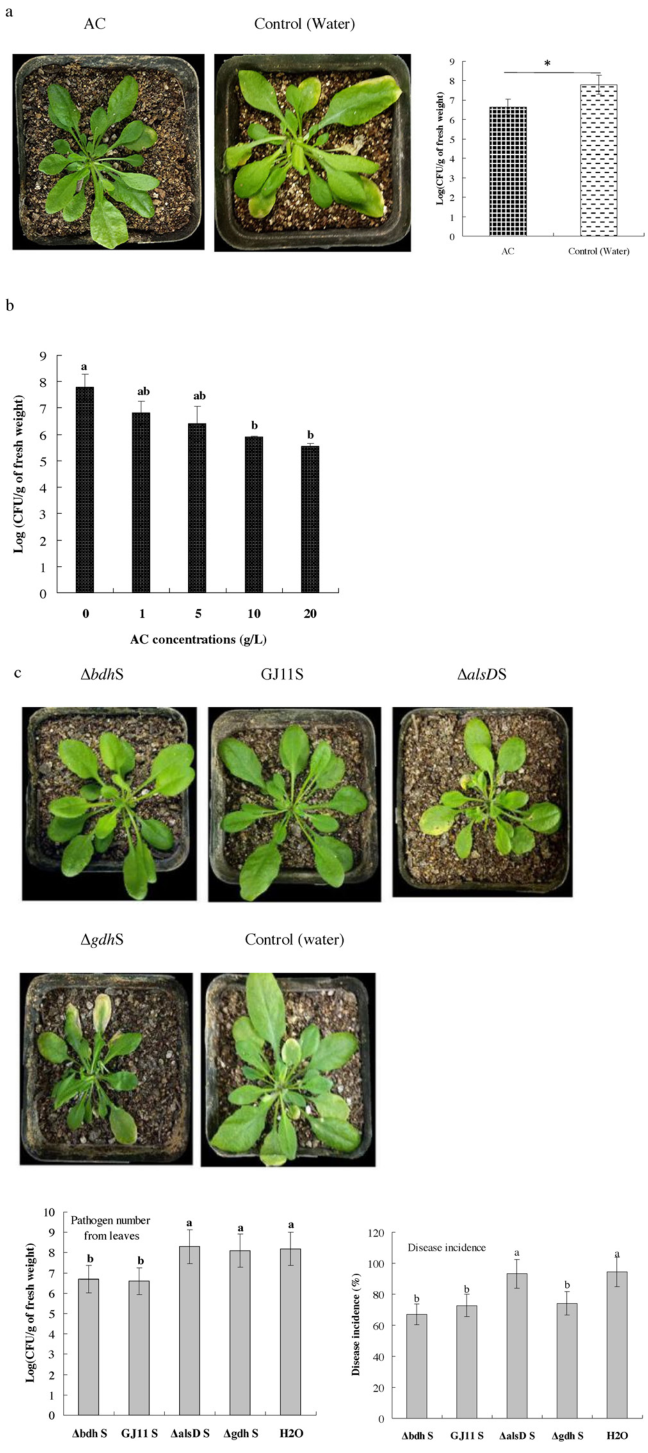

Fig. 3. Induction of ISR against *Pst* DC3000 in *A. thalianathe* plants. (a): Acetoin elicited ISR against *Pst* DC3000. The plants were treated with 10 g/L of acetoin or sterile water by root-drench. 3 days later, leaves were inoculated with *Pst* DC3000 at 1 × 10^8 CFU/mL, then the effect of acetoin on disease symptom was determined. Single star indicates significant difference ($p < 0.05$) between acetoin and water. **(b):** Effect of different doses of acetoin on triggering ISR in *A. thaliana*. The plants were irrigated with different concentrations of acetoin, then inoculated with *Pst* DC3000. Thereafter, the leaves were collected for quantifying *Pst* DC3000. Different letters indicate significant difference ($p < 0.05$) among treatments. **(c):** Induction of ISR against *Pst* DC3000 by GJ11 and mutant strains. Plants were treated with the broth supernatant of GJ11, GJ11Δ*bdh*, GJ11Δ*alsD* or GJ11Δ*gdh* by root-drench. 3 days later, leaves were inoculated with *Pst* DC3000 at 1 × 10^8 CFU/mL, then the effect of different treatments on disease symptom was determined. GJ11 (Δ*bdh*, Δ*gdh*, Δ*alsD*) S: Broth supernatant of GJ11 (GJ11Δ*bdh*, GJ11Δ*gdh*, GJ11). Different letters indicate significant difference ($p < 0.05$) among treatments. AC: acetoin. This experiment was repeated in triplicates, and N = 10 seedlings per group.

accumulation, callose deposition and enhanced enzymes activity related with defense response (Ahn et al., 2007). In order to know whether acetoin could induce the defence-related response in plants, the roots of *A. thaliana* were treated with acetoin, the broth supernatant of GJ11Δ*bdh* with high production of acetoin, or the broth supernatant of GJ11Δ*alsD* without production of acetoin by irrigation, then inoculated by *Pst* DC3000. The results showed treatment with acetoin, the broth supernatant of GJ11Δ*bdh* or the broth supernatant of GJ11Δ*alsD* but without *Pst* DC3000 inoculation, no H_2O_2 accumulation and callose deposition was observed in the leaves (Fig. 4a–c). Inoculation with pathogen alone could elicit H_2O_2 accumulation and callose deposition in plants even they were not pre-primed by acetoin, or the broth supernatant of bacterial strains (Fig. 4d). Further investigation found pretreatment with acetoin, or the broth supernatant of GJ11Δ*bdh* with high production of acetoin could elicit mild but effective plant immune response in the systemic tissues called "priming" (Kierul et al., 2015), in which the plants could rapidly response to pathogens infection (*e.g. Pst* DC3000) accompanying with H_2O_2 accumulation and callose deposition (Fig. 4f&g). On the contrary, the broth supernatant of GJ11Δ*alsD* without acetoin production could only induce the plants in weak "priming" state, thus weak H_2O_2 accumulation and callose deposition was observed after being infected by *Pst* DC3000 when compared to GJ11Δ*bdh* (Fig. 4e). The results suggested that acetoin and the bacterial strain GJ11Δ*bdh* with high production of acetoin can effectively elicit plants in a "priming" state, which is favorable for rapidly triggering a strong defense-related response such as H_2O_2 accumulation and callose deposition in plants once upon infected by pathogens.

3.6. Activity of defence-related enzymes in Arabidopsis induced by acetoin

The defence-related enzymes such POD, PPO, PAL and CAT are important for protecting plants from pathogens infection. The results showed irrigation with acetoin could enhance the POD activity in *Arabidopsis* leaves after 24 h, but there was no significant difference from the control (Left panel, Fig. 5a). However, once infected by *Pst* DC3000, the POD activity in leaves pre-treated with acetoin rapidly increased from 6 to 12 h, then decreased at 24 h, and increased again from 30 to 36 h (Right panel, Fig. 5a). In this period, the POD activity was significantly higher than the control (water) at two time points (6 h and 36 h post inoculation by *Pst* DC3000). The results suggested that pre-treatment with acetoin is favorable for increasing the POD activity to alleviate the toxic role of ROS, which are induced by pathogens infection and harmful both to pathogens and hosts.

Irrigation with acetoin could also increase the PPO activity in *Arabidopsis* leaves, but there was no significant difference from the control (Left panel, Fig. 5b). However, once infected by *Pst* DC3000, the PPO activity in *Arabidopsis* leaves pre-treated with acetoin rapidly

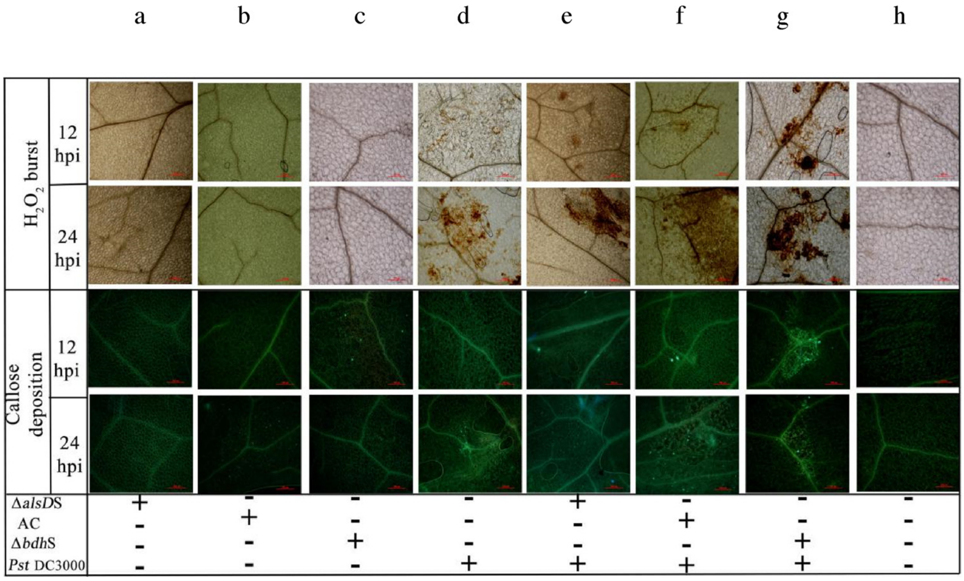

Fig. 4. ROS accumulation and callose deposition in the leaves of *A. thalianathe*. Plants were treated with acetoin, or the broth supernatant of GJ11Δ*bdh* or GJ11Δ*alsD*, then inoculated with *Pst* DC3000. After that, the leaves were collected for detecting ROS accumulation and callose deposition by histochemical staining. Δ*alsD*S and Δ*bdh*S: Broth supernatant of GJ11Δ*alsD* and GJ11Δ*bdh*, respectively. hpi: hours post-treatment with acetoin or broth supernatant **(a–c)**, hours post inoculation with *Pst* DC3000 **(d–g)**, and hours post-treatment with water **(h)**. AC: acetoin. This experiment was repeated in triplicates, and N = 10 seedlings per group.

increased from 6 h to 24 h, with a very significant difference from the control at 12 h and 24 h, respectively (Right panel, Fig. 5b). This result implied that pre-treatment with acetoin can also induce the rapid increase of PPO activity in plants upon infected by pathogens that is favorable for bio-synthesis of polyphenols to inhibit pathogens infection.

Irrigation of *Arabidopsis* with acetoin could also induce the change of PAL activity in leaves. After treatment with acetoin, the PAL activity in *Arabidopsis* leaves increased from 12 h, and was significantly higher than the control at 48 h (Left panel, Fig. 5c). Upon infected by *Pst* DC3000, the PAL activity in leaves pre-treated with acetoin rapidly increased from 6 h to 30 h, and was significantly higher than the control at 12 h. Thereafter, it varied similar to the control until the end of experiment (Right panel, Fig. 5c). The results verified that pre-treatment with acetoin could also induce the increase of PAL activity in leaves that was favorable for bio-synthesis of phytoalexin to inhibit pathogens infection in plants. Differently, treatment with acetoin could not significantly increase the CAT activity in leaves when compared to the control, even the plants were challenge with *Pst* DC3000 after treatment with acetoin (Fig. 5d).

3.7. Acetoin-induced defence response was dependent on H_2O_2

H_2O_2 accumulation could be observed in the leaves pre-treated with acetoin upon infected by pathogens (Fig. 4). The acetoin-treated *Arabidopsis* was inoculated by *Pst* DC3000 mixed with CAT or ASC to investigate the role of ROS such as H_2O_2 in the defense-response induced by acetoin. The results showed the plants pre-treated with acetoin following inoculated by *Pst* DC3000 mixed with CAT or ASC showed more obvious symptom than the ones without CAT or ASC (Fig. 6a), indicating CAT or ASC could weaken the acetoin-triggered ISR in *Arabidopsis*. Also, the acetoin-induced H_2O_2 accumulation and callose deposition was weakened in the *Pst* DC3000-inoculated leaves co-administered with CAT or ASC (Fig. 6a). Logically, the CFU number of *Pst* DC3000 was much higher in the leaves co-treated with acetoin and ASC or CAT when compared to the ones treated with acetoin only (Fig. 6b). Thereby, the defense-response induced by acetoin could be inhibited by cleaning H_2O_2 with ASC or CAT in plants. This result verified that the acetoin-induced defense response was dependent on H_2O_2, consistent with the CAT activity that was not enhanced in the leaves of *Arabidopsis* treated with acetoin (Fig. 5d).

The H_2O_2 content in leaves was quantitatively determined after treatment with acetoin. The results showed the H_2O_2 content in *Arabidopsis* leaves treated with acetoin rapidly increased to the maximum level at 12 h that was significantly higher than the control, thereafter rapidly decreased to the lowest level at 24 h, showing a "burst" of H_2O_2 in the leaves (Up and left panel, Fig. 6c). Inoculation with *Pst* DC3000 could enlarge the H_2O_2 burst in *Arabidopsis* leaves treated with acetoin, in which the H_2O_2 content rapidly increased from 6 to 24 h that was significantly higher than the control in this period (Up and right panel, Fig. 6c). The control group also showed a burst of H_2O_2 from 24 h to 48 h after inoculation by *Pst* DC3000, but was much later than the group pre-treated with acetoin (Up and right panel, Fig. 6c). The result suggested pre-treatment with acetoin could induce H_2O_2 burst in plants at an early stage once infected by pathogens that was favorable for eliciting an effective defense response against pathogens infection.

The H_2O_2 content in *Arabidopsis* was also quantitatively detected after treatment with the bacterial broth. After treatment with the broth supernatant of GJ11, GJ11Δ*bdh* or GJ11Δ*alsD*, the H_2O_2 content increased rapidly in leaves at 12 h that was significantly higher than the control (Down and left panel, Fig. 6c). Thereafter, the H_2O_2 content was still significantly higher in *Arabidopsis* treated with the broth of GJ11Δ*bdh* or GJ11 than the control. Conversely, the H_2O_2 content in *Arabidopsis* treated with the broth of GJ11Δ*alsD* was significantly lower than the control (Down and left panel, Fig. 6c). Comparison with GJ11, the mutant strain GJ11Δ*bdh* with higher production of acetoin was stronger to induce the H_2O_2 production in plants, with a significant difference at 12 and 24 h, respectively (Down and left panel, Fig. 6c). *Pst* DC3000 infection could enhance the H_2O_2 production in *Arabidopsis* leaves treated with GJ11Δ*bdh*, in which the H_2O_2 burst was significantly different from the control at several time points such as 6, 12, 24, 36 and 48 h (Down and right panel, Fig. 6c). Also, the pathogen infection could enhance the H_2O_2 production in *Arabidopsis* leaves pre-treated with GJ11, but the H_2O_2 burst was only observed at 36 h (Down and right panel, Fig. 6c). Overall, the H_2O_2 burst induced by GJ11 was weaker than the one induced by GJ11Δ*bdh*. Also, there was H_2O_2 burst in the plants pre-treated with GJ11Δ*alsD* at 36 h, but it was much weaker than the one induced by GJ11 or GJ11Δ*bdh*, even weaker than the control (Down and right panel, Fig. 6c). The results suggested that acetoin or the bacterial strains with high production of acetoin could induce plants to produce H_2O_2 in a "priming" state, in which the plants could rapidly response to pathogens infection.

3.8. Colonization of GJ11 and GJ11Δbdh in plant rhizosphere and root

Both GJ11 and GJ11Δ*bdh* could well colonize in the rhizosphere and

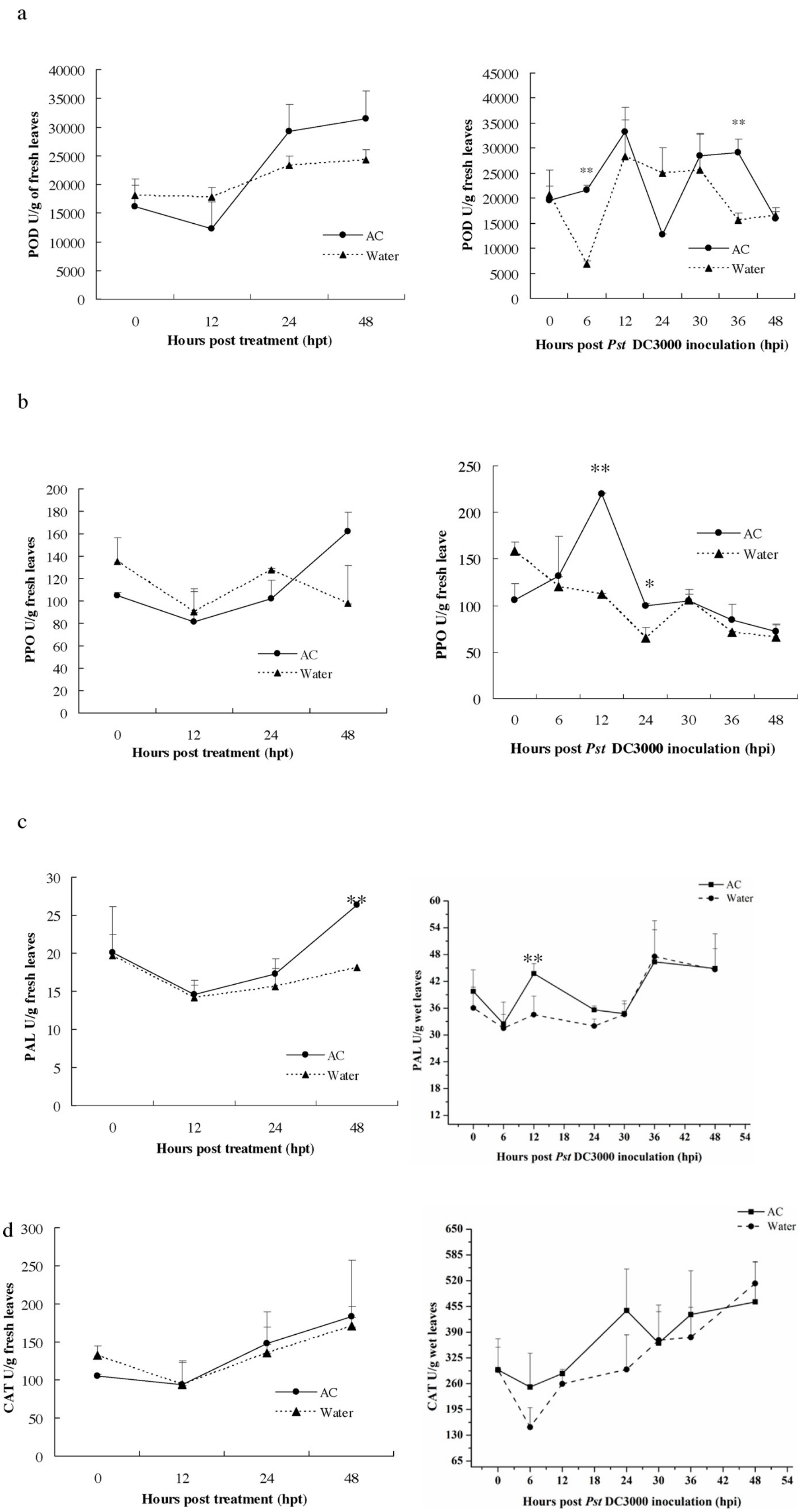

Fig. 5. Activity of defense-related enzymes in leaves of *A. thalianathe*. Activity of POD **(a)**, PPO **(b)**, PAL **(c)** and CAT **(d)** in the leaves treated with acetoin by irrigation **(Left panel)**, and followed with inoculation by *Pst* DC3000 **(Right panel)**. hpt, hours post-treatment with acetoin; hpi, hours post-inoculation with *Pst* DC3000. Star indicates significant difference ($p < 0.05$) from the control, and double stars mean very significant difference ($p < 0.01$) from the control. This experiment was repeated in triplicates, and N = 10 seedlings per group.

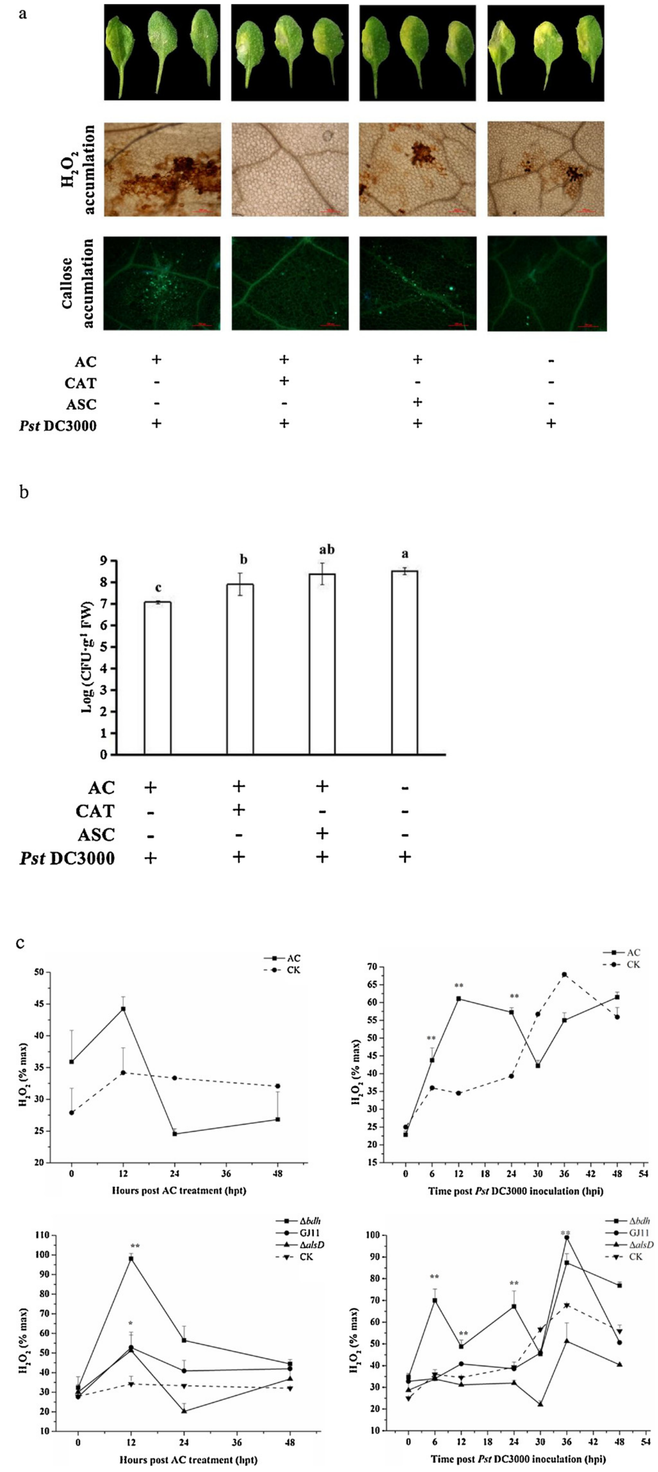

Fig. 6. Defense response triggered by acetoin is dependent on H_2O_2. (a): Disease symptom and defensive responses including H_2O_2 accumulation and callose deposition in leaves. Leaf symptom was observed 3 days post inoculation by *Pst* DC3000. H_2O_2 burst and callose deposition was determined at 24 h post inoculation by *Pst* DC3000. AC: acetoin; CAT: catalase; ASC: ascorbic acid. **(b):** *Pst* DC3000 in the leaves of *A. thalianathe*. Logarithmic numbers of *Pst* DC3000 recovered from leaves were quantified 3 days post inoculation. Different letters indicate a significant difference ($p < 0.05$) among treatments. **(c):** Variance of H_2O_2 content in the leaves of *A. thalianathe* treated with acetoin, or the broth supernatant of GJ11Δ*alsD* and GJ11Δ*bdh* **(Left panel)**, followed with inoculation by *Pst* DC3000 **(Right panel)**. AC: acetoin; GJ11, Δ*alsD* and Δ*bdh*: Broth supernatant of GJ11, GJ11Δ*alsD* and GJ11Δ*bdh*, respectively; CK: irrigation with water as control. Single star indicates a significant difference ($p < 0.05$) from the control, and double stars mean a very significant difference ($p < 0.01$) from the control. This experiment was repeated in triplicates, and N = 10 seedlings per group.

root of *A. thalianathe*. The number of GJ11 and GJ11*bdh* was about 10^7-10^8 CFU/g in the rhizospheric soil, and 10^4-10^5 CFU/g in the root of *A. thalianathe* (Fig. S2). Both of these two strains could be lastly observed in the rhizospheric soil and root at high levels for more than 40 days. Thereby, both GJ11 and GJ11Δ*bdh* could well colonize the plant rhizosphere as PGPRs, and enter into the plant as endophytes.

4. Discussion

PGPRs can trigger ISR to enhance the plant resistance to pathogens infection. Generally, PGPRs produce microbial - associated molecular patterns (MAMPs) such as bacterial lipopolysaccharide, flagellin, exopolysaccharides, 2,4-diacetylphloroglucinol, N-acyl homoserine lactones, lipopeptides, *etc.*, to induce plant immune response (Jiang et al., 2016; Pieterse, 2009; Zhang and Zhou, 2010; Zipfel and Felix, 2005). Moreover, many PGPRs can also release VOCs to mediate plant - microbe interactions. Recently, the bacterial VOCs such as 2,3-BD are generally considered as MAMPs to trigger ISR in plants (Ryu et al., 2004). In this study, pharmacological application of acetoin could trigger ISR in plants. To confirm the role of acetoin in triggering ISR under biological conditions, several *B. velezensis* GJ11 mutant strains including GJ11Δ*alsD*, GJ11Δ*bdh* and GJ11Δ*gdh* were constructed, respectively. The mutant strain genetically blocked in acetoin and 2,3-BD synthesis (GJ11Δ*alsD*) was compared with the wild-type strain to examine the effect of acetoin on triggering ISR in plants. In this comparison, GJ11Δ*alsD* was greatly impaired in eliciting plant ISR, whereas the wild-type strain successfully elicited a plant defense response against pathogen infection, indicating the bacterial acetoin plays an important role in plant protection. This was further confirmed by the mutant strain GJ11Δ*bdh* with high production of acetoin that triggered much stronger ISR than the wild-type strain GJ11. Moreover, GJ11Δ*bdh* could trigger stronger ISR than the mutant strain GJ11Δ*gdh* with high production of *D*-2,3-BD, also suggested that acetoin is stronger to trigger ISR in plants.

Acetoin is a product of carbon overflow metabolism (Wang et al., 2012). Many *Bacillus* strains can produce acetoin but does not reach the functional levels required for the beneficial action in plants (Chung et al., 2016). Here, in order to use acetoin for bio-control of plant diseases at low costs, the metabolic engineering was performed to improve the acetoin production in *B. velezensis*. Our metabolic engineering approaches facilitated to increase acetoin production by knockout of *bdh* gene to block the pathway from acetoin to 2,3-BD in GJ11, and the resultant mutant strain GJ11Δ*bdh* could trigger stronger ISR and defense response than the wild-type strain. Addition to *bdh*, the *gdh* gene was also reported to be responsible for bio-synthesis of *D*-2,3-BD in *B. licheniformis* (Qiu et al., 2016). Therefore, we deleted *gdh* in *B. velezensis*, and found knockout of *gdh* could not improve acetoin production but increase *D*-2,3-BD production in broth. This was different from the literature, possibly due to the different *Bacillus* species used. Thereby, GDH was not responsible for synthesis of 2,3-BD in G11, and after deletion of *gdh*, NADH could not be used for synthesis of glycerol hence it was possibly used for synthesis of 2,3-BD in *B. velezensis* that resulted in an increase of *D*-2,3-BD production in GJ11Δ*gdh* at several time points (Fig. 1). The gene *budC* was also reported to catalyze the conversion between acetoin and *meso*-2,3-BD in *B. licheniformis* (Qi et al., 2014), but it could not be found in the genomic DNA of *B. velezensis*, consistent with the fact that mainly *D*-2,3-BD rather than *meso*-2,3-BD was found in GJ11 (Fig. 1). Thereby, the genetic engineering strain about *budC* was not performed in this study.

Isomeric form is very important for the activity of 2,3-BD to trigger ISR in plants. For example, *D*-2,3-BD is strong to trigger ISR while *L*-2,3-BD can not induce plant immunity (Chung et al., 2016; Han et al., 2006; Ryu et al., 2003, 2004). Certain *Bacillus* strains such as *B. licheniformis* produce the stereoisomers of 2,3-BD (*e.g.* *L*-2,3-BD and *meso*-2,3-BD) that are not activate for triggering ISR (Wang et al., 2012). Here, *B. velezensis* GJ11 selectively produced the active 2,3-BD (*D*-2,3-BD) and acetoin, thereby it showed a good activity in triggering ISR. The mutant strain GJ11Δ*gdh* could also produce *D*-2,3-BD, hence it was also effective on triggering ISR in plants.

In this study, we report the air-borne chemicals of acetoin and 2,3-BD released from *B. velezensis*, triggered ISR in *Arabidopsis* seedlings. We found that the defense-response induced by acetoin was dependent on H_2O_2. In fact, many bacteria can produce 2,3-BD and acetoin under low oxygen concentrations to provide an alternative electron sink for the regeneration of NAD when aerobic respiration is limited. Pyruvate is catalyzed to acetoin, then acetoin is reduced to 2,3-BD by NADH (Kim et al., 2011). Therefor, acetoin has a stronger oxidizability than 2,3-BD. It is interesting to note that the enzymes for generating 2,3-BD and acetoin have been identified in several crops such as tobacco, corn, carrot, and rice cultures (Forlani et al., 1999; Ryu et al., 2004). We postulated that acetoin might deprive the plant's NADH to form 2,3-BD *in vivo* that was favorable for forming H_2O_2 in the plant cells due to lack of NADH (Ederli et al., 2006). This was consistent with our observation that treatment with acetoin could elicit a burst of H_2O_2 in plants. The burst of H_2O_2 can directly kill pathogens, as well act as a signal molecule to induce plant defense response (Ryu et al., 2003).

H_2O_2 acts as a double-edged sword that can kill pathogens but is also harmful to hosts. For alleviating the toxic role of H_2O_2, plants generally enhance the anti-oxidant enzymes activity such as POD *in vivo*. Here, after pre-treatment with acetoin, the plants were primed in a state with strong response to pathogens infection. After infection by *Pst* DC3000, the plants pre-treated with acetoin fast produced H_2O_2 to kill the pathogens, as well as enhanced the activity of POD to alleviate H_2O_2 injury (Figs. 5 & 6). The CAT activity was also enhanced in the plants pre-treated with acetoin after inoculation by Pst DC3000 at several time points such as 6 h and 24 h, but there was no significant difference from the control (Fig. 5d). Thereby, there was only a slight increase of CAT activity in plants. This might be beneficial for the hosts to alleviate H_2O_2 toxicity to some degree without significant disturbance of its role to kill the pathogens, and act as a signal molecule to elicit the immune response (*e.g.* the enhanced PAL and POD activity) in plants. As a result, the enzymes activity of PAL and PPO were both enhanced in the acetoin-treated plants for synthesis of phytoalexin and polyphenols to inhibit pathogens infection (Fig. 5).

After recognizing microbes, the immune response is induced to a state called as "priming" (Beckers, 2006; Schwessinger and Ronald, 2012; Wu et al., 2014). Here, the plants treated with acetoin showed an obvious priming state, in which the mild immune response was observed such as a slight increase of H_2O_2 production, callose deposition, and enzymes activity related with defence response. However, once upon infected by pathogens, the plants could rapidly response to the infection accompanying with a significant increase of ROS (*e.g.* H_2O_2) production, callose deposition, and enzymes activity related with defense response. If the immune response is further enhanced, the plants will go into hypersensitive cell death response (HR) that is favorable for enhancing the resistance towards pathogens infection (Jones and Dang, 2006). Here, acetoin could not only trigger ISR but also elicit HR, indicating acetoin can induce different plant defense responses. Activation both of HR and ISR can enhance the defense-response towards pathogens infection (Pieterse et al., 2014).

2,3-BD is considered important for the bacterial colonization in the plant rhizosphere. Yi et al. (2016) reported that *B. subtilis* mutant strain without production of 2,3-BD was easier to be eliminated in the rhizosphere than the wild-type strain, while the strain with 2,3-BD overexpressor could lastly maintain at roots. In this study, even the mutant strain GJ11Δ*bdh* only produced a minor 2,3-BD, it was still persistent in the plant root and rhizosphere similar to the wild-type strain. It may be explained by the fact that GJ11Δ*bdh* could produce a high level of acetoin although only a minor 2,3-BD was produced by this strain, hence we postulated that acetoin could play a similar role for the bacterial colonization in the plant root and rhizosphere as 2,3-BD.

In conclusion, we verified acetoin is a main component of VOCs produced by *B. velezensis*, which can induce a priming state (*e.g.* H_2O_2 burst and callose deposition) in plants. This can augment the response to pathogens attack by a manner depended on H_2O_2. This capacity to induce disease resistance by triggering ISR is expected to work in concert with the colonization efficiency, making *B. velezensis* GJ11, especially the genetic engineering strain *B. velezensis* GJ11Δ*bdh* with high production of acetoin, excellent candidates of biocontrol agent in the future. To our knowledge, this is the first report to use the genetically engineered bacterial strain with high production of acetoin for triggering more effective and stronger ISR in plants.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126297.

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