Plant Biotechnol Rep (2012) 6:381–390
DOI 10.1007/s11816-012-0235-2

ORIGINAL ARTICLE

A novel gibberellin 2-oxidase gene *CaGA2ox1* in pepper is specifically induced by incompatible plant pathogens

Yeon Lee · Young-Cheol Kim · Soo Yong Kim · In-Jung Lee · Doil Choi · Kyung-Hee Paek · Hye Sun Cho · Suk Yoon Kweon · Jeong Mee Park

Received: 3 May 2012 / Accepted: 16 May 2012 / Published online: 10 June 2012

Abstract Phytohormone balance is increasingly recognized as central to the outcome of plant–pathogen interactions. Differential screening for genes induced by a non-host pathogen in pepper plants (*Capsicum annuum*) identified a putative gibberellin 2-oxidase gene, *CaGA2ox1*. Analysis of the deduced amino acid sequence of *CaGA2ox1* showed 53 and 50 % amino acid identity to *Pisum sativum* PsGA2ox2 and *Arabidopsis* AtGA2ox6, respectively. Expression in pepper plants of *CaGA2ox1* was preferentially increased in response to non-host pathogen inoculation and during the host resistance response. *CaGA2ox1* expression increased following treatment with salicylic acid and ethephon (albeit with different induction patterns), but remained unchanged following treatment with methyl jasmonate and abscisic acid. The gene product of *CaGA2ox1* is predicted to catalyze the metabolism of GA_4, and does so in recombinant *E. coli* extracts. Further PEG-mediated transient expression studies showed that CaGA2ox1 fused with soluble modified green fluorescent protein localized to the cytosol in chili pepper protoplasts. Interestingly, the transcript level of *CaGA2ox1* was not affected by treatments of either pepper with bioactive GA_{4+7} or paclobutrazol, an inhibitor of GA biosynthesis. Taken together, these results provide the first evidence that a GA 2-oxidase, which is important in GA metabolism, may also play a role in plant defense signaling and plant–microbe interactions.

Keywords *CaGA2ox1* · Chili pepper (*Capsicum annum*) · Gibberellin (GA) · Non-host pathogen

Introduction

When challenged with pathogen invasion, plants activate various defense responses, including activation of signal transduction pathways leading to expression of defense-related genes [such as pathogenesis-related (PR) genes], activation of the hypersensitive response (HR), and synthesis of phytoalexins with antimicrobial activity (Dangl et al. 1996). The major signals involved in plant defense responses are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Dong 1998; Reymond and Farmer 1998; Guo and Ecker 2004), which together fine-tune the expression of biotic stress-responsive genes in plants (Hammond-Kosack and Parker 2003). However, the interactions between these signaling networks and the signaling pathways mediated by other phytohormones, such as gibberellin (GA) and auxin, have generally remain unresolved.

GA is a tetracyclic diterpenoid phytohormone important in several aspects of plant development, including seed germination, leaf expansion, stem elongation, flowering,

Electronic supplementary material The online version of this article (doi:10.1007/s11816-012-0235-2) contains supplementary material, which is available to authorized users.

Y. Lee · Y.-C. Kim · S. Y. Kim · H. S. Cho · S. Y. Kweon · J. M. Park (✉)
Green Bio Research Center, KRIBB, Daejeon 305-333, Republic of Korea
e-mail: jmpark@kribb.re.kr

I.-J. Lee
Division of Plant Bioscience, Kyungpook National University, Daegu 702-701, Republic of Korea

D. Choi
Department of Plant Science, Seoul National University, Seoul 151-921, Republic of Korea

K.-H. Paek
School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea

and seed development (Sun and Gubler 2004). During the last decade, much progress has been made to understand the mechanism of GA signaling. It is well known that GAs promote plant growth by inducing the degradation of the DELLA family of nuclear transcription factors. The mechanism of GA-induced disappearance of the growth-restraining DELLA proteins (Jiang et al. 2007) is highly conserved between dicots and monocots (Fleet and Sun 2005). Recently, a role for DELLA proteins has been proposed in the responses of plants to pathogen attack: loss-of-function mutations in DELLA proteins improve the resistance of plants to some pathogens through induction of a salicylic acid-dependent defense pathway (Robert-Seilaniantz et al. 2007; Navarro et al. 2008). The authors indicate that GAs are able to regulate SA biosynthesis during plant responses to pathogens. However, neither the mechanism of GA action on defense responses nor how GA modulates changes in metabolism in response to pathogen attack is known. Recently, Yang et al. (2008) reported that a GA-deactivating enzyme called EUI (Elongated Uppermost Internode) is involved in basal disease resistance against bacterial and fungal pathogens in rice. The *Eui* gene encodes a P450 that deactivates biologically active GAs through GA 16α,17-epoxidation (Zhu et al. 2006). Thus far, it is unclear whether GA 16α,17-epoxidation is a common GA deactivation reaction in other plant species.

The level of bioactive GA in plants is regulated by the relative rates of its synthesis and deactivation. The important steps in the biosynthetic pathway of bioactive GA are the formation of C_{19}-GA through the oxidation of C_{20}-GA, and the 3β-hydroxylation of C_{19}-GA. The 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase and GA 3-oxidase, catalyze these respective steps in GA biosynthesis (Hedden and Phillips 2000; Olszewski et al. 2002). Another step regulating the endogenous level of GA is the deactivation of the bioactive GA forms GA_1 and GA_4 and their respective precursors, GA_9 and GA_{20}, by 2β-hydroxylation. This step is catalyzed by another 2-oxoglutarate-dependent dioxygenase, GA 2-oxidase (GA2ox), in the cytoplasm (Thomas et al. 1999; Hedden and Phillips 2000; Olszewski et al. 2002). In *Arabidopsis*, garden pea (*Pisum sativum*), rice (*Oryza sativa*), and spinach (*Spinacia oleracea*), *GA2ox*s are encoded by small gene families (Schomburg et al. 2003; Lester et al. 1999; Sakamoto et al. 2001; Lee and Zeevaart 2002). Examination of the *GA2ox* gene from various plants has focused on its role in development, particularly in plant growth (Sakamoto et al. 2001; Lee and Zeevaart 2002; Schomburg et al. 2003; Busov et al. 2003; Ogawa et al. 2003; Frisse et al. 2003; Wang et al. 2004; Lee and Zeevaart 2005). Loss-of-function mutations of *PsGA2ox1*, *AtGA2ox7*, and *AtGA2ox8* result in a hyperelongated phenotype (Martin et al. 1999; Schomburg et al. 2003). Similarly, in poplar (*Populus tremula* × *Populus alba*), overexpression of *PtaGA2ox1* causes a stumpy phenotype characterized by extremely short internodes and dark-green leaves with a stiff, leathery texture (Busov et al. 2003). These phenotypes were also observed with overexpression of *AtGA2ox6*, which is directly regulated by AGL-15, a member of the MADS box transcription factor family (Wang et al. 2004).

Using cDNA microarray analysis, we isolated a putative GA 2-oxidase gene, *CaGA2ox1*, from pepper plants (*Capsicum annuum* L. cv. Bukang) challenged with the non-host bacterium *Xanthomonas axonopodis* pv. *glycines* 8ra (*Xag*8ra, soybean pustule pathogen; Hwang et al. 1992). Based on expression analysis of *CaGA2ox1*, we suggest that CaGA2ox1 in pepper specifically responses to biotic stress. To our knowledge, this is the first report that a GA 2-oxidase, which catalyzes the 2-oxidation of C_{19} GAs, is involved in plant defense responses.

Materials and methods

Plant materials and pathogen inoculation

Chili pepper (*Capsicum annuum* L. cv. Bukang) and bell pepper [*C. annum* L. cv. Early Calwonder (ECW) 30R; *bs1*/*bs1*, *bs2*/*bs2*, *Bs3*/*Bs3*] plants were used in the current study. Peppers were grown in a plant growth room at 24 ± 1 °C using a photoperiod of 16 h light and 8 h dark. Healthy and well-expanded leaves from 6-week-old plants were used for pathogen inoculation and various chemical treatments. The bacterial pathogens used for inoculation were *Xanthomonas axonopodis* pv. *glycines* 8ra (*Xag*8ra), a soy bean pustule pathogen (Hwang et al. 1992), and the pepper bacterial spot pathogens *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) race 1 (*avrBs3*) and race 3 (Kousik and Ritchie 1996). Bacterial infiltration was performed by syringe infiltration of bacterial suspensions (approximately 4 x 10^8 cfu/ml). Inoculated leaves were harvested at the indicated time points and used as the source of RNA for further analysis.

Chemical treatments

For the various chemical treatments, detached chili pepper leaves were soaked in solutions with dissolved final concentrations of 5 mM salicylic acid, 5 mM ethephon (ET), or 100 μM methyl jasmonic acid (MeJA) in sterile water. All treated leaves were harvested at the indicated time points and frozen in liquid nitrogen until further analyses were conducted.

RNA gel blot analysis

Total RNA was extracted from leaf tissues of pepper plants according to Choi et al. (1996). RNA (20 μg) isolated from

leaf tissues treated with pathogens or chemicals was separated by electrophoresis on formaldehyde-containing 1 % agarose gels and transferred onto nylon membranes (Amersham, USA). Hybridizations were performed using the full-length cDNA probe of *CaGA2ox1* labeled with ^{32}[P]-dCTP according to the method of Church and Gilbert (1984). Membranes were washed twice with 2× SSC/0.1 % SDS at room temperature for 5 min, once with 1× SSC/0.1 % SDS at room temperature for 15 min, and once with 0.1× SSC/0.1 % SDS at room temperature for 5 min. The membranes were visualized using a BAS-1800II phosphoimager (Fuji Photo Film, Japan). The pepper cDNA control probes in these experiments, *CaPR1* and *CaPIN-II*, were isolated previously (Yi et al. 2004 and Shin et al. 2001).

Plant treatments with GA_{4+7} and paclobutrazol (PBZ)

Either 50 ng of GA_{4+7} or PBZ were first dissolved in 0.5 ml of ethanol and then diluted with distilled water to a 50 ml volume. These chemicals were applied to the pot (379-ml plastic pots, Horticulture Nursery Media Low soil mixture; Pu-nong, Korea) of 2-week-old chili pepper plants for a week. The plants were incubated in the growth chamber at 24 °C in conditions as above. Changes in growth were observed and photographed 7 days after treatment. Experiments were performed twice using independently grown pepper seedlings.

RT-PCR and Q-RT-PCR analyses

Material for RNA analysis was ground in liquid nitrogen, and total RNA was isolated using TRI reagent according to the manufacturer's protocol (MRC, Canada). Reverse transcription was performed using 2 μg of total RNA and SuperScript reverse transcriptase II (Invitrogen). The gene expression levels of various genes were determined by semi-quantitative PCR and quantitative RT-PCR using specific primer sets (Tables S1 and S2). Quantitative PCR was run on a Light cycler system (BIO-RAD) according to the manufacturer's recommendations with the following conditions: 1 cycle of 15 min at 95 °C, and 40 cycles of 20 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. The specificity of the amplifications was verified by melting curve analysis of the PCR products at the end of each experiment. Actin was used as an internal standard.

Subcellular localization

The full-length *CaGA2ox1* cDNA without the termination codon was prepared by polymerase chain reaction (PCR) using the N-terminal specific primer (5′-GGGTCTAGAGTCTAATATGGTAGTGG-3′) containing an *Xba*I site and the C-terminal specific primer (5′-TTTGGATCCCATTGGGCATGGTTCG-3′) containing a *Bam*HI site. The PCR-amplified product was cloned into the pGEM-T easy vector (Promega, Madison, USA), digested with *Xba*I and *Bam*HI, and purified by agarose gel electrophoresis. The resulting fragment was fused to the coding region of soluble-modified green fluorescent protein (smGFP; David and Vierstra 1996). The *CaGA2ox1*:smGFP fusion construct was introduced into protoplasts, prepared from chili pepper leaves, by polyethylene glycol-mediated transformation (Park et al. 2001). The fluorescent micrographs of protoplasts were taken using a confocal laser scanning microscope (Carl Zeiss LSM510, Germany) 24 h after transformation. The filter sets used were BP505-530 (excitation 488 nm, emission 505–530 nm) and LP650 (excitation 488 nm, emission 650 nm; Carl Zeiss) to detect GFP and chlorophyll autofluorescence, respectively.

Expression of *CaGA2ox1* in *E. coli* and assay for GA 2-oxidase activity

The coding region of *CaGA2ox1* was amplified by PCR with the primers F1, 5′-GTCTAATATGGTAGTGGCAACTC-3′ and R2, 5′-GGTAGCTAAGACTGCTAAGTACTGC-3′. The resulting PCR product was cloned into pGEM T-easy vector (Promega), digested with *Eco*R1 and *Xho*1 and then cloned into the corresponding restriction sites of the *pET28a* vector (Clontech, USA). Either this full length cDNA clone (*pET CaGA2ox1*) or *pET* empty vector were transformed into *Escherichia coli* strain BL21pLysis. Cell lysate was prepared as described by Lee and Zeevaart (2002). The presence of CaGA2ox1 fusion protein in the cell lysate was confirmed by western blot analysis with an anti-His antibody (Invitrogen). Transformation with pGEX-*SoGA2ox1* was used as a positive control for the enzyme activity assay (Lee and Zeevaart 2002). Enzyme assays with recombinant CaGA2ox1 were performed with [^{14}C]-labeled GA_4. The assays and methods for product identification were conducted as previously described (Lee and Zeevaart 2002; Schomburg et al. 2003).

Results and discussions

Isolation of *CaGA2ox1*

In order to identify a wide variety of genes induced in pepper plants during resistance to non-host pathogens, cDNA microarray analysis was performed with probes obtained from *Xag*8ra-infected hot pepper leaves. *Xag*8ra is not a pathogen of pepper, but does elicit an HR in pepper leaves as well as induce the expression of a number of *PR* genes (Lee et al. 2004). As a result of the microarray

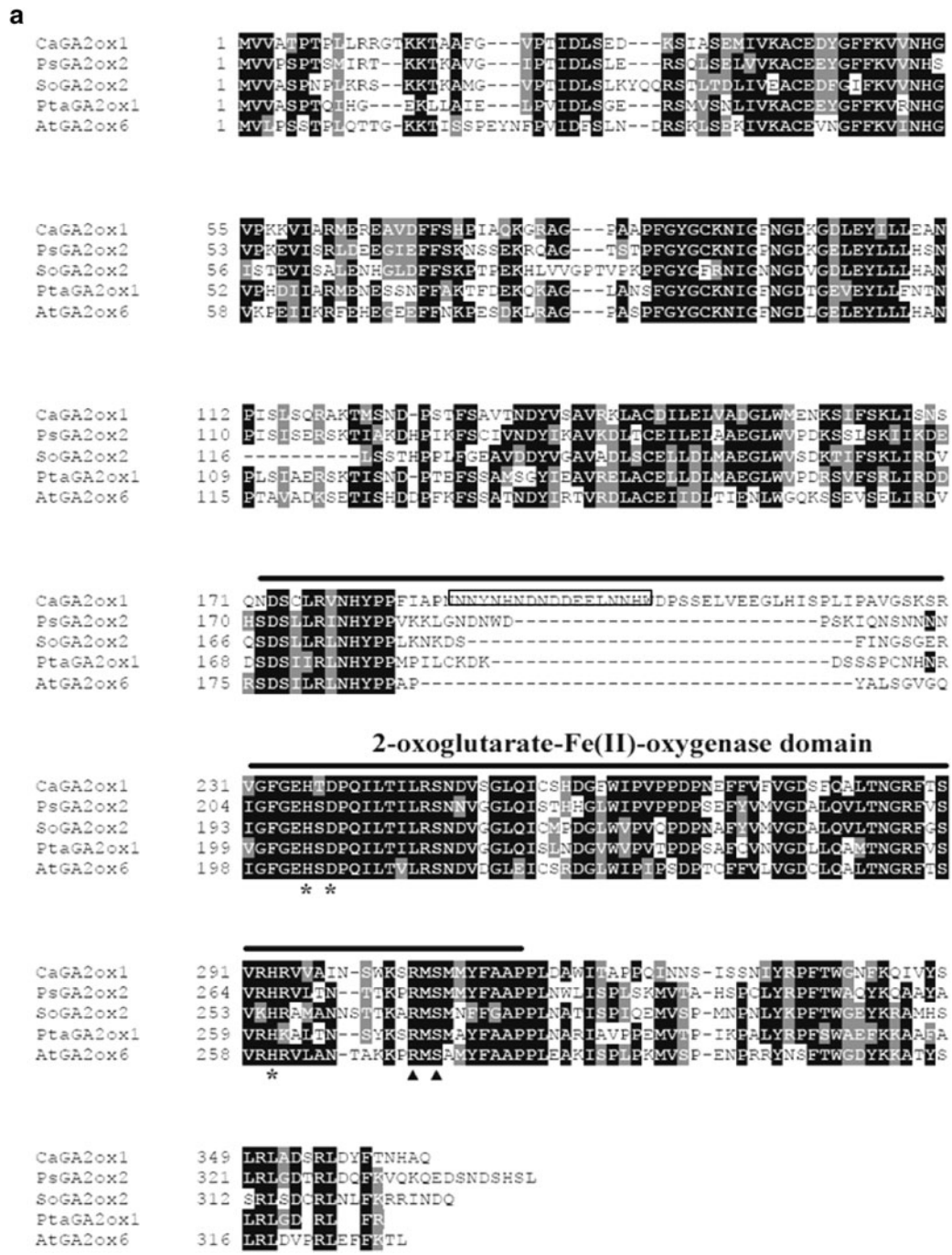
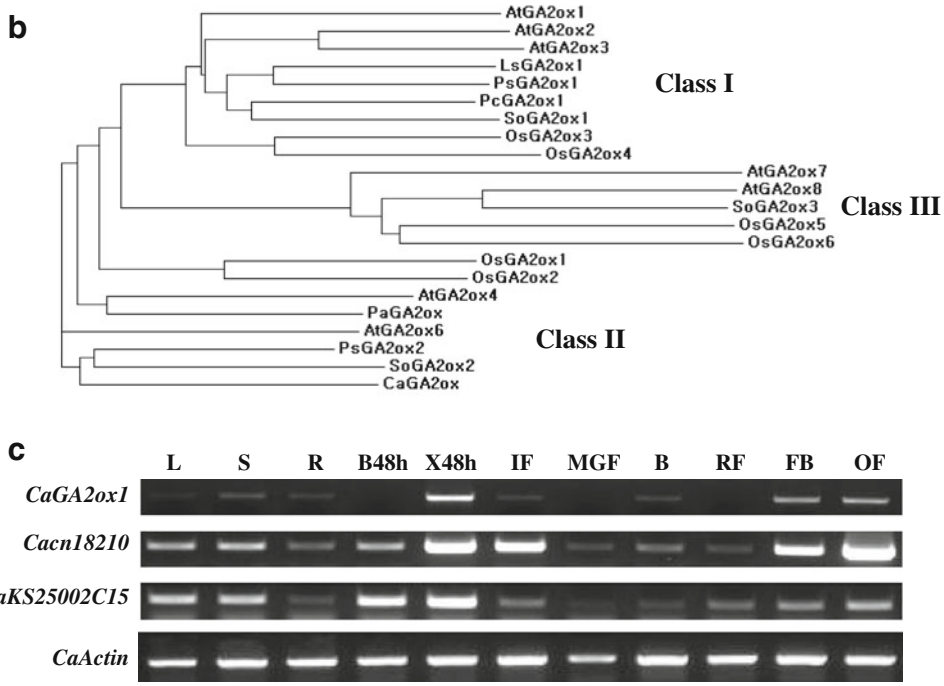


Fig. 1 Comparison of amino acid sequences of CaGA2ox1 and other GA2oxs and tissue-specific expression of *CaGA2ox1*. **a** Sequence alignment of the deduced amino acid sequence of *CaGA2ox1* with four closely related GA2ox genes. The *asterisks* indicate the three amino acid residues forming the iron-binding site. The *closed triangles* show the putative 2-oxoglutarate binding site. The *boxed region* indicates unique asparagine-rich amino acid sequences of CaGA2ox1. **b** Phylogenetic relationship of *CaGA2ox1* with other plant GA2oxs using ClustalW (http://www.ebi.ac.uk/clustalw/). The GenBank, DDBJ, EMBL, or NCBI accession numbers and consensus IDs from the pepper EST database (http://genepool.kribb.re.kr/new) for each protein are: *CaGA2ox1* (DQ465393), AtGA2ox1 (CAB41007), AtGA2ox2 (CAB41008), AtGA2ox3 (CAB41009), AtGA2ox4 (AAG51528), AtGA2ox6 (AAG00891), AtGA2ox7 (AAG50945), AtGA2ox8 (CAB79120), LsGA2ox1 (BAB12442), OsGA2ox1 (BAB40934), OsGA2ox2 (BAC16751), OsGA2ox3 (BAC16752), OsGA2ox4 (AAU03107), OsGA2ox5 (BAC10398), OsGA2ox6 (CAE03751), PcGA2ox1 (CAB41036), PsGA2ox1 (AAF08609), PsGA2ox2 (AAD45424), PtaGA2ox1 (AAQ93035) SoGA2ox1 (AAN87571), SoGA2ox2 (AAN87572), and SoGA2ox3 (AAX14674). **c** Tissue-specific expression of the *CaGA2ox1* gene. Total RNA was extracted from leaf (*L*), stem (*S*), root (*R*), at 48 h after 1 mM $MgCl_2$ treatment (*B48h*), 48 h after *Xag* 8ra inoculation (*X48h*), immature fruit (*IF*), mature green fruit (*MGF*), breaker (*B*), red fruit (*RF*), flower bud (*FB*), and open flower (*OF*). RT-PCR analysis was carried out using the primers described in Supplemental Table S1

Fig. 1 continued



analysis, a gene encoding a key enzyme in gibberellin metabolism, a gibberellin 2-oxidase, designated *CaGA2ox1*, was isolated. The *CaGA2ox1* gene encodes a putative protein that consists of 364 amino acids with a molecular mass of 40 kDa (Fig. 1a). The deduced amino acid sequence of *CaGA2ox1* shares 53, 51, and 50 % identity, respectively, with *Pisum sativum* PsGA2ox2 (Lester et al. 1999), poplar PtaGA2ox1 (Busov et al. 2003), and *Arabidopsis thaliana* AtGA2ox6 (Wang et al. 2004). Alignment of the amino acid sequence of CaGA2ox1 with those of other GA 2-oxidases confirmed that it belonged to this class of enzymes, as it contains characteristic sequences that are conserved within the GA 2-oxidase class, including the 2-oxoglutarate-binding site (arginine and serine residues) and the iron-binding site (histidine and aspartic acid residues; Thomas et al. 1999). However, the amino acid sequence of the *CaGA2ox1* protein shows unique asparagine-rich amino acid sequences (located at positions 188–206) that are different from other GA dioxygenases (Fig. 1a).

A phylogenetic tree was generated using the deduced amino acid sequences of *CaGA2ox1* with the GA2oxs from other plants and other GA2ox homologs, which were the most closely related proteins available at the time these studies were initiated from the pepper EST database (EST ID: *cacn18210*, *CaKS25002C15*; http://genepool.kribb.re.kr/new/; Fig. 1b, S1). The GA 2-oxidase family was distributed among three different clades, with class I and II enzymes catabolizing C_{19}-GAs and class III enzymes catalyzing the 2β-hydroxylating reaction of C_{20}-GAs (Lee and Zeevaart 2005). The phylogenetic tree analysis indicates that *CaGA2ox1* belongs to the class II enzymes with *PsGA2ox2*, *PaGA2ox*, *AtGA2ox6*, *and SoGA2ox2*. *CaGA2ox1* may be a GA 2-oxygenase that has the conserved regions of GA-dioxygenase activity and generates 2β-hydroxylated C_{19}-GAs, such as the bioactive GA_4 and GA_1.

In various plants, *GA2ox* genes are expressed in diverse tissues. To examine the steady-state expression level of *CaGA2ox1* in various organs of the hot pepper plant, total RNA was extracted from roots, stems, leaves, fruit, flower bud, and open flower. The level of *CaGA2ox1* expression was most abundant in the flower tissues, while low level expression was detected in other tissues (Fig. 1c). In contrast, the other pepper *GA2ox* homologs, *CaKS25002C15*, and *cacn18210*, were more abundant than *CaGA2ox1* in all tissues in which they were expressed. The tissue-specific expression pattern of *cacn18210* was similar to *CaGA2ox1* and was induced by *Xag*8ra inoculation (Fig. S4).

Expression of *CaGA2ox1* gene in HR

To determine the expression pattern of the *CaGA2ox1* gene during the non-host plant–pathogen interaction, transcript levels of the *CaGA2ox1* gene were monitored over time by RNA gel blot analysis (Figs. 2a, S2). Hot pepper leaves infected with *Xag*8ra exhibited biphasic *CaGA2ox1* transcripts level increases. *CaGA2ox1* transcripts were strongly induced within 1 h of pathogen infection, were markedly decreased after 6 h, and then were highly induced again at

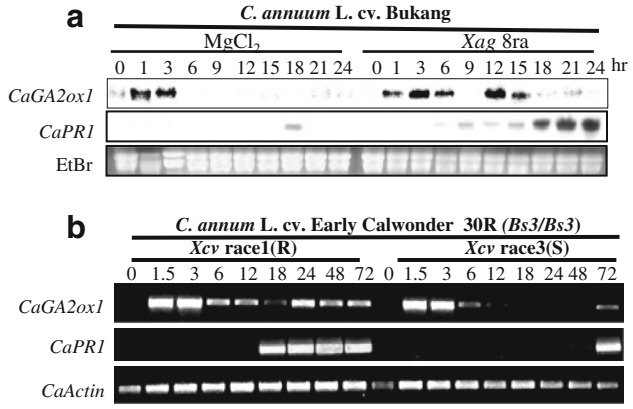


Fig. 2 Expression of the *CaGA2ox1* gene in pepper plants inoculated with bacterial pathogens. **a** Chili pepper plants (cv. Bukang) were inoculated with a non-host bacterial pathogen, *Xag*8ra. **b** Expression patterns of the *CaGA2ox1* gene in cv. ECW30R plants challenged with the bell pepper plant bacterial spot pathogens, *Xav* race 1 (*avrBs3*) or race 3. Total RNA was isolated from pepper leaves harvested at the indicated time points

12 h. When leaves were infiltrated with the buffer control, rapid accumulation of *CaGA2ox1* mRNA was also detected from 1 to 3 h after $MgCl_2$ infiltration, but not induced again until 24 h. This transient upregulation in both treatments partly reflected the effects of mechanical stress caused by infiltration. RT-PCR analysis of wound-treated pepper RNA samples disclosed that this early transient induction of *CaGA2ox1* was due to the wounding effect of infiltration (Fig. S2). In contrast, expression of *CaPR1*, a positive control for inoculation of *Xag*8ra, was first detected at 9 h after bacterial infection and gradually increased thereafter.

In addition to the non-host pathogen resistance response, the expression of *CaGA2ox1* in *R*-gene-mediated disease resistance was studied (Fig. 2b). The bell pepper cultivar ECW-30R, which contains the dominant allele *BS3*, was used. ECW-30R plants are resistant to *Xanthomonas axonopodis* pv. vesicatoria race 1 (*Xav* 1), which possesses the *avrBS3* avirulence gene, but are susceptible to the *Xav* race 3 (*Xav* 3) strain, which causes bacterial spot disease (Kousik and Ritchie 1996). RT-PCR analysis was carried out using a primer set specific for *CaGA2ox1* and *CaPR1* (Table S1). As shown in Fig. 2b, strong and early (1.5–3 h after inoculation) induction of *CaGA2ox1* transcripts was detected in both compatible and incompatible interactions with *Xav*. In the incompatible interaction, however, induced *CaGA2ox1* mRNA levels were detected until 18 h post-inoculation and then its transcripts were re-induced at 24 h and kept until 72 h; by contrast, early time induction of *CaGA2ox1* rapidly disappeared at 12 h in compatible interaction and a later weak induction appeared 72 h after *Xav* 3 with expression significantly lower than that in the leaves of ECW-30R-infected *Xav* 1. The expression pattern of *CaPR1* was also examined as a positive control for *Xav* inoculation. Specific induction of *CaGa2ox1* during incompatible interactions between pepper and bacterial pathogens (Fig. 2a, b) suggests the possibility that *CaGA2ox1* is functional in pathogen-induced defense responses in pepper plants.

Expression of *CaGA2ox1* in response to various chemicals

To investigate the expression of *CaGA2ox1* in response to endogenous defense-related signals, detached hot pepper leaves were treated with 5 mM SA, 5 mM ethephon, or 100 μM MeJA before isolation of mRNA at each time point. As shown in Fig. 3, the addition of ET and SA dramatically increased *CaGA2ox1* gene expression, although the expression pattern was different in response to each treatment. *CaGA2ox1* transcripts began to accumulate as early as 6 h after SA treatment, reached their maximum level at 24 h, and continued to be expressed at this level at 48 h after treatment. The ET treatment more effectively induced the early expression of *CaGA2ox1*. *CaGA2ox1* mRNA was strongly expressed within 6 h of ET treatment and diminished by 48 h. On the other hand, the expression of *CaGA2ox1* was not affected by MeJA treatment, the defense chemical mediating responses to mechanical wounding (Reymond and Farmer 1998) and which induced the strong expression of the chili pepper *proteinase inhibitor* within 12 h after MeJA treatment (Fig. 2b; Shin et al. 2001). The *CaPR1* gene was detected within 24 h of ET and SA treatments. Recently, it has been proposed that abscisic acid (ABA), which is an important signaling molecule in the abiotic stress responses in plants, is also involved in the regulation of DELLA protein degradation in the plant's growth response to high salt stress (Achard et al. 2006). The *CaGA2ox1* mRNA was not induced with the application of exogenous ABA (Fig. S2). Together, these results indicate that the induction of *CaGA2ox1* expression following pathogen inoculation is affected by SA and/or ET accumulation and, moreover, that SA and ethylene indirectly affect GA metabolism.

CaGA2ox1 protein has a GA 2-oxidase activity and is localized to the cytoplasm

One mechanism regulating the GA biosynthetic pathway may be the subcellular compartmentalization of the pathway. To investigate the cellular distribution of *CaGA2ox1*, the entire *CaGA2ox1* cDNA sequence was fused to a soluble-modified green fluorescent protein (smGFP; David and Vierstra 1996) under the control of the CaMV 35S promoter. The resulting construct was introduced into pepper protoplasts by polyethylene glycol-mediated transformation (Park et al. 2001). Localization of the fusion

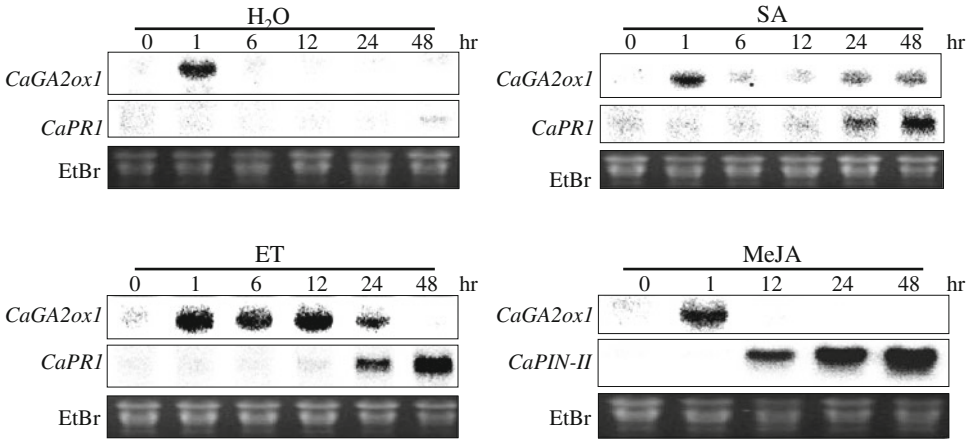


Fig. 3 Expression of the *CaGA2ox1* gene in detached leaves treated with chemicals related to various stress responses. Total RNA was extracted from detached pepper leaves treated with buffer, as negative control, 5 mM SA, 5 mM ethephon (ET), or 100 μM MeJA at the indicated time points. RNA (20 μg) was separated by electrophoresis, blotted, and hybridized with a ^{32}P-dCTP-labeled full-length cDNA of *CaGA2ox1*. The expression of *CaPR1* in SA- or ET-treated leaves and *CaPIN-II* in MeJA-treated leaves was monitored as positive controls. The 25S and 18S rRNA bands in ethidium bromide-stained gels are shown as loading controls. One typical result from three independent experiments was presented

protein was determined using confocal fluorescence microscopy. The CaGA2ox1:smGFP fusion protein was strongly expressed in pepper protoplasts and was localized to the cytoplasm of the cells (Fig. 4a), indicating that *CaGA2ox1* may function in the cytosol.

To test whether *CaGAa2ox1* encodes a gene product with GA 2-oxidase activity, the full-length coding region was expressed in *Escherichia coli* (Fig. S3). The recombinant fusion protein was present in the soluble portion of the induced cell lysate. As described previously, the crude extract was incubated with C^{14}-labeled GA_4, a C_{19}-GA (Lee and Zeevaart 2002; Schomburg et al. 2003). As shown in Fig. 4b, cell lysates expressing CaGA2ox1 fusion protein in *E. coli* converted C^{14}-GA_4 to GA_{34}, indicating that *CaGA2ox1* encodes a functional GA 2-oxidase. There are two *Arabidopsis* genes that sort into the class II clade, *GA2ox4* and *GA2ox6*. GA2ox4 has been shown to act exclusively as a C_{19}-GA 2-oxidase (Jasinski et al. 2005), while GA2ox6 was similarly reported to have activity on GA_4 and GA_1 (Wang et al. 2004). The activity of CaGA2ox1 on C_{20}-GAs and other C_{19}-GAs was not tested.

Treatment with biologically active GA does not affects *CaGA2ox1* expression

Thomas et al. (1999) reported that GA 2-oxidase is involved in maintenance of the concentration of biologically active GA in plant tissues. The effects of GA_{4+7} and PBZ, an inhibitor of GA biosynthesis (Kitahata et al. 2005; Toh et al. 2008), on the transcript levels of *CaGA2ox1* and its homologs were examined using real-time quantitative RT-PCR (Q-RT-PCR; Fig. 5) with the inclusion of the GA biosynthetic genes *GA20ox* and *GA3ox* (Gallego-Giraldo et al. 2008) positive controls. *CaKS25002C15* and *cacn18210* transcript levels were decreased in plants treated with PBZ but were highly increased upon GA_{4+7} application (Fig. 5b). However, *CaGA2ox1* transcripts were not significantly changed under PBZ or GA_{4+7} treatments. The expression of the GA-biosynthesizing *CaGA20ox1* highly increased with PBZ application, but not with GA or buffer treatment. *CaGA3ox1* transcript levels were decreased in pepper plants treated with GA_{4+7}, but were increased with PBZ application. Taken together, these results indicated that the expression of *CaKS25002C15* and *cacn18210* are under feedback regulation by the level of biologically active GA, whereas the expression of *CaGA2ox1* is not.

Conclusion

GA2ox is the enzyme that catalyzes the deactivation of bioactive GAs, a reaction that is important in the regulation of endogenous GA levels (Hedden and Phillips 2000; Olszewski et al. 2002). Expression studies of the three *Arabidopsis* GA 2-oxidases revealed that two of these enzymes, *AtGA2ox1* and *AtGA2ox2*, were most abundant in the inflorescence and developing siliques, whereas the third enzyme, *AtGA2ox3*, could not be detected in any tissue (Thomas et al. 1999). This expression pattern is consistent with a role for GA 2-oxidases in reducing GA levels in seeds to promote dormancy. In pepper, transcripts of *CaGA2ox1* were not detectable by RNA gel blot analysis and were only detected by RT-PCR in flower tissue after

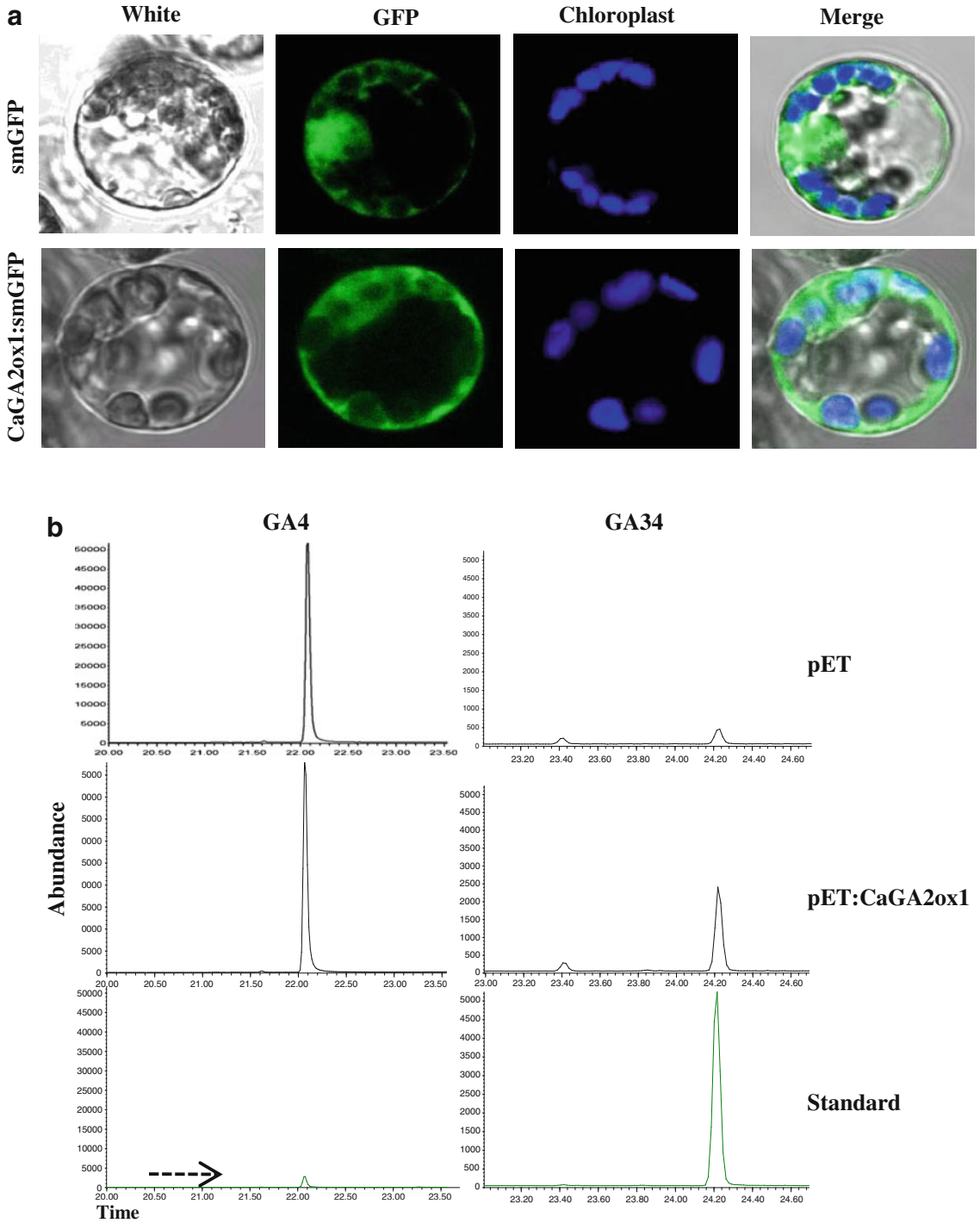


Fig. 4 **a** Subcellular localization of the *CaGA2ox1* gene product. The *CaGA2ox1* coding region was fused to the coding region of smGFP and introduced into pepper protoplasts by polyethylene glycol-mediated transformation. Fluorescent micrographs of protoplasts were taken using a confocal laser scanning microscope (Carl Zeiss LSM510, Germany) at 24 h after transformation. **b** Oxidase activity against GA4 of recombinant CaGA2ox1. The *pET*:*CaGA2ox1* recombinant fusion protein expressed in BL21*pLysS* cell line was incubated with C^{14}-labeled GA4. The conversion of GA4 to GA34 was confirmed by HPLC

>38 cycles. However, as shown in Fig. 2a, b, expression of *CaGA2ox1* was greatly increased in leaves inoculated with bacterial pathogens. In addition, the transcript level of *CaGA2ox1* was not affected by treatment with active GAs, even though it encodes a GA 2-oxidase shown to inactivate GA_4 (Fig. 4b). These results indicate that *CaGA2ox1* might

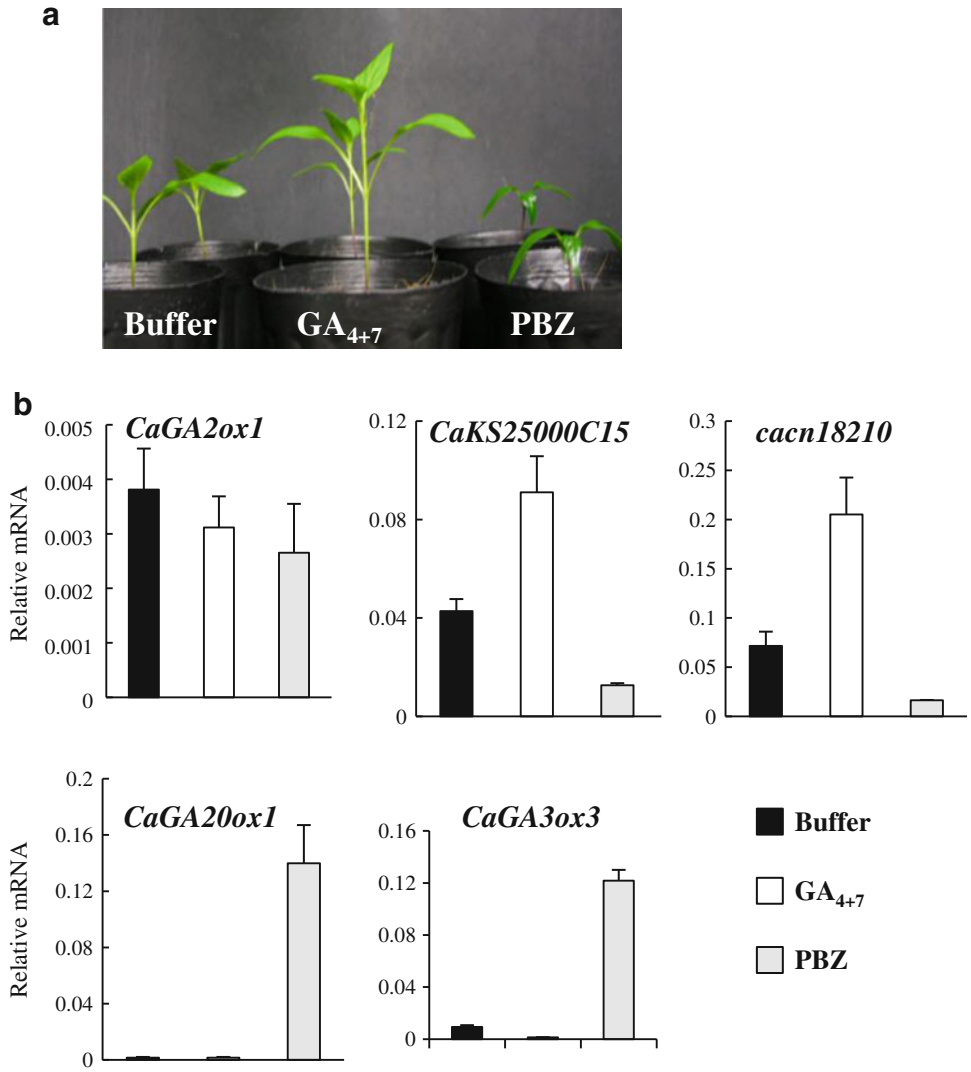


Fig. 5 The effects of GA_{4+7} and PBZ on the level for *CaGA2ox1*. **a** Phenotype of pepper treated with buffer, 50 μM GA_{4+7} or 50 μM PBZ. **b** The expression of *CaGA2ox1*, homologous GA2oxs, and GA biosynthesis genes such as GA20 oxidase and GA3 oxidase in response to GA_{4+7} and PBZ applications to pepper plants. The levels of *CaGA2ox1* and GA biosynthesis genes were examined using real-time quantitative RT-PCR. Independent experiments were repeated one more time with similar results

has a role specifically in disease resistance responses in pepper plants. In summary, through the isolation of the gene *CaGA2ox1*, which encodes a putative GA oxidizing enzyme, we speculated that two independent, pathogen-activated phytohormonal signaling pathways (SA and ET) regulate plant defense responses through induction of GA metabolism. Further studies in pepper plants silenced for multiple GA 2-oxidase homologs and global gene expressions using a pepper cDNA microarray will be useful in elucidating the roles of GA 2-oxidases. Moreover, directly observing GAs contents during plant defense responses may help to explain the function of GAs and coordinate interactions of GAs with other phytohormones in plant disease resistance responses.

Acknowledgments This work was financially supported by the Cabbage Genomics assisted breeding supporting Center funded by Ministry for Food, Agriculture, Forestry and Fisheries of the Korean Government (to J.M.P.) and partly the KRIBB initiative program (to J.M.P.).

References

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van der Straeten D, Peng JR, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 311:91–94

Busov VB, Meilan R, Pearce DW, Ma C, Rood SB, Strauss SH (2003) Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. Plant Physiol 132:1283–1291

Choi D, Kim HM, Yun HK, Park JA, Kim WT, Bok SH (1996) Molecular cloning of a methallothionein-like gene from *Nicotiana glutinosa* L. and its induction by wounding and tobacco mosaic virus infection. Plant Physiol 112:353–359

Church GM, Gilbert M (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Dangl JL, Dietrich RA, Richberg MH (1996) Death don't have no mercy: cell death programs in plant–microbe interactions. Plant Cell 8:1793–1807

David SJ, Vierstra RD (1996) Soluble derivatives of green fluorescent protein (GFP) for use in *Arabidopsis thaliana*. Weeds World 3:43–48

Dong X (1998) SA, JA, ethylene, and disease resistance in plants. Curr Opin Plant Biol 1:316–323

Fleet CM, Sun TP (2005) A DELLAcate balance: the role of gibberellin in plant morphogenesis. Curr Opin Plant Biol 8:77–85

Frisse A, Pimenta MJ, Lange T (2003) Expression studies of gibberellin oxidase in developing pumpkin seeds. Plant Physiol 131:1220–1227

Gallego-Giraldo L, Ubeda-Toma S, Gisbert C, García-Martínez L, Moritz T, López-Díaz I (2008) Gibberellin homeostasis in tobacco is regulated by gibberellin metabolism genes with different gibberellin sensitivity. Plant Cell Physiol 49:679–690

Guo H, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol 7:40–49

Hammond-Kosack KE, Parker JE (2003) Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. Curr Opin Biotechnol 14:177–193

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci 5:523–530

Hwang I, Lim SM, Shaw PD (1992) Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. *glycines*. J Bacteriol 174:1923–1931

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu L, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr Biol 15:1560–1565

Jiang C, Gao X, Liao L, Harberd NP, Fu X (2007) Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in *Arabidopsis*. Plant Physiol 145:1460–1470

Kitahata N, Saito S, Miyazawa Y (2005) Chemical regulation of abscisic acid catabolism in plants by cytochrome P450 inhibitors. Bioorg Med Chem 13:4491–4498

Kousik CS, Ritchie DF (1996) Race shift in *Xanthomonas campestris* pv. vesicatoria within a season in field-grown pepper. Phytopathology 86:952–958

Lee DJ, Zeevaart JAD (2002) Differential regulation of RNA levels of gibberellin dioxygenases by photoperiod in spinach. Plant Physiol 130:2085–2094

Lee DJ, Zeevaart JAD (2005) Molecular cloning of GA 2-oxidase3 from spinach and its ectopic expression in *Nicotiana sylvestris*. Plant Physiol 138:243–254

Lee S, Kim SY, Chung E, Joung YH, Pai HS, Hur CG, Choi D (2004) EST and microarray analyses of pathogen-responsive genes in hot pepper (*Capsicum annuum* L.) non-host resistance against soybean pustule pathogen (*Xanthomonas axonopodis* pv. glycines). Funct Integr Genomics 4:196–205

Lester DR, Ross JJ, Smith JJ, Elliott RC, Reid JB (1999) Gibberellin 2-oxidation and the SLN gene of *Pisum sativum*. Plant J 19:65–73

Martin DN, Proebsting WM, Hedden P (1999) The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol 121:775–781

Navarro L, Bari R, Achard P, Lison P, Nemri A, Harberd NP, Jones JDG (2008) DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr Biol 18:650–655

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during *Arabidopsis* seed germination. Plant Cell 15:1591–1604

Olszewski N, T-P Sun, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell 14:S61–S80

Park JM, Park CJ, Lee SB, Ham BK, Shin R, Paek KH (2001) Overexpression of the tobacco *Tsi* gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell 13:1035–1046

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol 1:404–411

Robert-Seilaniantz A, Navarro L, Bari R, Jones JD (2007) Pathological hormone imbalances. Curr Opin Plant Biol 10:372–379

Sakamoto T, Kobayashi M, Itoh H, Tagiri A, Kayano T, Tanaka H, Iwahori S, Matsuoka M (2001) Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol 125:1508–1516

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JAD, Amasino RM (2003) Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell 15:151–163

Shin R, Lee GJ, Park CJ, Kim TY, You JS, Nam YW, Paek KH (2001) Isolation of pepper mRNAs differentially expressed during the hypersensitive response to tobacco mosaic virus and characterization of a proteinase inhibitor gene. Plant Sci 161:727–737

Sun T-p, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. Annu Rev Plant Biol 55:197–223

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA 96:4698–4703

Toh S, Imamura A, Watanabe A (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in *Arabidopsis* seeds. Plant Physiol 146:1368–1385

Wang H, Caruso LV, Downie AB, Pery SE (2004) The embryo MADS domain protein AMOUS-like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. Plant Cell 16:1206–1219

Yang Dl, Li Q, Deng YW, Lou YG, Wang MY, Zhou ZX, Zhang YY, He ZH (2008) Altered disease development in the eui mutants and Eui overexpressors indicates that gibberellins negatively regulate rice basal disease resistance. Mol Plant 1:528–537

Yi SY, Kim JH, Joung YH, Lee S, Kim WT, Yu SH, Choi D (2004) The pepper transcription factor *CaPF1* confers pathogen and freezing tolerance in *Arabidopsis*. Plant Physiol 136:2862–2874

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z (2006) ELONGATED UPPERMOST INTERNODE encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. Plant Cell 18:442–456