Short title: Freezing tolerance and fertility GA 2-oxidases

The class-III gibberellin 2-oxidases AtGA2ox9 and AtGA2ox10 contribute to cold stress tolerance and fertility

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One-sentence summary: Two multifunctional class-III gibberellin 2-oxidases are examined; one contributes to freezing tolerance, the other regulates seed production.

Keywords: gibberellins, AtGA2ox9, AtGA2ox10, catabolism, Arabidopsis, cold stress tolerance, fertility
ABSTRACT

Many developmental processes in plants are regulated by gibberellin hormones. Gibberellin homeostasis is achieved via complex biosynthetic and catabolic pathways. Gibberellin catabolic enzymes include GA 2-oxidases that are classified into three classes. Members of class-III GA 2-oxidases typically act on GA precursors containing a C_{20}-skeleton. Here, we identified two further members of this class of GA 2-oxidases, namely *AtGA2ox9* and *AtGA2ox10*, in the *Arabidopsis* (*Arabidopsis thaliana*) genome. Both genes encode enzymes that have functional similarities to *AtGA2ox7* and *AtGA2ox8*, which are class-III GA 2-oxidases that 2ß-hydroxylate C_{20}-GAs. Previously unknown for GA 2-oxidases, *AtGA2ox9* performs 2α-hydroxylation of C_{19}-GAs and harbors putative desaturate activity of C_{20}-GAs. Additionally, *AtGA2ox9* and *AtGA2ox10* exhibit GA 20-oxidase activity. *AtGA2ox9* oxidizes carbon-20 to form tricarboxylic acid C_{20}-GAs, whereas *AtGA2ox10* produces C_{19}-GA_{9}. *AtGA2ox9* transcript levels increase after cold treatment and *AtGA2ox10* is expressed mainly in the siliques of Arabidopsis plants. *Atga2ox9* loss-of-function mutants are more sensitive to freezing temperatures, whereas *Atga2ox10* loss-of-function mutants produce considerably more seeds per siliques than wild-type plants. We conclude that, in Arabidopsis, *AtGA2ox9* and *AtGA2ox10* contribute to freezing tolerance and regulate seed production, respectively.
INTRODUCTION

Bioactive gibberellins (GAs) are signaling molecules essential for plant growth and responses to environmental conditions (Yamaguchi, 2008; Sun, 2010; Colebrook et al., 2014). Gibberellins regulate plant developmental processes, including flowering, fertilization, and embryo growth (Mutasa-Göttgens and Hedden, 2009; Pimenta Lange and Lange, 2006; Martínez-Bello et al., 2015; Pimenta Lange et al., 2020). A common reaction to abiotic stresses (e.g. cold, salt, osmotic stress, or touch) is the reduction of GA levels, and consequently, decrease of plant growth. There is increasing evidence that GA-inactivation is an integral part of the plant hormonal response pathway and that, in particular, catabolic GA 2-oxidases are the main players within this process (Colebrook et al., 2014; Pimenta Lange and Lange, 2006; Pimenta Lange and Lange, 2015; Achard et al., 2008). GA 2-oxidases belong to soluble 2-oxoglutarate-dependent-dioxygenases (2ODDs) that are encoded by multigene families (Lange and Pimenta Lange, 2020; Hedden and Phillips, 2015).

Lee and Zeevaart (2005) proposed three structural classes of GA 2-oxidases: Class-I and -II enzymes utilize C19-GAs whereas class-III enzymes accept C20-GAs as substrates. However, exceptions to this rule were published recently: Class-I GA 2-oxidases from cucumber (Cucumis sativus) and bread wheat (Triticum aestivum) are also capable of oxidizing C20-GAs whereas bread wheat class-III GA 2-oxidases also converts C19-GAs (Pimenta Lange and Lange, 2013; Pearce et al., 2015). In Arabidopsis, nine genes code for GA 2-oxidases, four of which belong to the class-III enzymes, including the two characterized herein (Fig. 1A and Supplemental Fig. S1).

In this study, we report two further GA 2-oxidases from Arabidopsis, designated AtGA2ox9 and AtGA2ox10, that are structurally related to class-III enzymes and that, consequently, both hydroxylate C20-GA12 to GA110 (2ß-hydroxylation; Fig. 1A). In addition, AtGA2ox9 catalyzes the conversion of other C20-GA precursors (including GA15, GA24, and GA25) further to putative mono-unsaturated GA46, which includes oxidations at carbon-2ß and carbon-20. Moreover, this enzyme oxidizes the C19-GAs GA9 and GA4 to GA40 and GA47, respectively (2α-hydroxylated products, Fig. 1A). As a side reaction, AtGA2ox10 also catalyzes oxidations at carbon-20 by two sequential conversions of C20-GA15 to C19-GA9 via GA24. We demonstrate that AtGA2ox9 is involved in acquiring freezing tolerance and that AtGA2ox10 contributes to plant growth and seed production in Arabidopsis.
Fig. 1. Enzymatic properties of class-III GA 2-oxidases in Arabidopsis. A. Scheme of the reactions catalyzed by the two identified multifunctional enzymes AtGA2ox9 (At5g58660) and AtGA2ox10 (At3g47190), which are illustrated in bold characters. C19-GAs are highlighted by a blue background, whereas all others are C20-GAs. B. HPLC-radiochromatograms of products from incubations of recombinant AtGA2ox9 and AtGA2ox10 with the C20-GAs 14C-GA12, -GA15, -GA24, and -GA25, and the C19-GAs 14C-GA9 and -GA4 as the substrates. Radiochromatograms with C19-GAs are in blue. Products were identified by GC-MS (Supplemental Table S1). Compound a, putative dihydro-GA110; compound b, putative dihydro-GA46.
RESULTS

Cloning of *AtGA2ox9* and *AtGA2ox10* from Arabidopsis. In a phylogenetic analysis, Porco et al. (2016) identified two putative 2-oxoglutarate dependent dioxygenases, At5g58660 and At3g47190, in the Arabidopsis genome that are related to the previously identified class-III GA 2-oxidases AtGA2ox7 and AtGA2ox8 (Schomburg et al., 2003). To investigate their enzymatic activities, we first cloned respective cDNA molecules in the pET101/D-TOPO expression vector. Total RNA, derived from Arabidopsis four-week-old shoots and siliques of 35-day-old plants, was used to clone At5g58660 (designated *AtGA2ox9*) and At3g47190 (designated *AtGA2ox10*), respectively. Positive clones were sequenced on both strands and the DNA sequences obtained for both genes are identical to the respective ones from the Arabidopsis genome (Supplemental Figs. S1, S2). The two previously uncharacterized GA 2-oxidases AtGA2ox9 and AtGA2ox10 consist of amino-acid sequences related to the previously described class-III GA 2-oxidases (Schomburg et al., 2003; Supplemental Figs. S1, S2). Several putative class-III related proteins also exist in other plant species, including cucumber and rice (*Oryza sativa*; Supplemental Fig. S1), but their enzymatic properties remain to be investigated.

*AtGA2ox9* and *AtGA2ox10* GA 2-oxidases are multifunctional. Both of the AtGA2ox9 and AtGA2ox10 recombinant enzymes 2ß-hydroxylated the C20-GA precursor GA12 and produced GA110 (Fig. 1, Supplemental Table S1). Moreover, AtGA2ox9 was shown to harbor activities previously unknown for GA 2-oxidases; it hydroxylates the C19-GAs GA9 and GA4 at 2α-position to form GA40 and GA47, respectively. Both products were identified conclusively by their KRIIs and mass spectra, which differ from respective 2ß-hydroxylated products (Pimenta Lange et al., 2020; Supplemental Table S1). Additionally, AtGA2ox9 and AtGA2ox10 were found to oxidize GA15 at C-20 position to GA24 (Fig. 1, Supplemental Tables S1, S2). Both enzymes also oxidized GA24, the next step of the GA biosynthetic pathway. AtGA2ox10 formed the C19-GA GA9 and AtGA2ox9 produced the C20-tricarboxylic acid GA25 that are typical products of anabolic and catabolic GA 20-oxidase activities, respectively (Lange and Pimenta Lange, 2020). Compound c, an unstable intermediate product from GA15 of AtGA2ox9, appeared to be converted non-enzymatically during derivatization to GA25 (Supplemental Table S2). AtGA2ox9 further oxidized GA25 at the C-2ß position to GA46 (Fig. 1, Supplemental Tables S1, S2) and also likely harbored desaturase activity as its reaction products from GA110 and GA46 represented, from their
mass spectra, mono-unsaturated GAs (compounds a and b, respectively; Fig. 1, Supplemental Tables S1, S2).

The previously identified class-III GA 2-oxidases AtGA2ox7 and AtGA2ox8 were shown to 2ß-hydroxylate GA12 (Schomburg et al., 2003). It was reported that AtGA2ox7 also accepts other C20-GA precursors, including GA15 and GA24, but the resulting products were not identified (Magome et al., 2008). By reinvestigating their enzymatic properties, we found that AtGA2ox7 and AtGA2ox8 hydroxylate GA12 to GA110 confirming the results of Schomburg et al. (2003) (Fig. 1A, Supplemental Fig. S3, Supplemental Table S1). Additionally, here we show that AtGA2ox7 converts C20-GAs GA15, GA24, and GA25 to 2ß-hydroxylated GA15, 2ß-hydroxylated GA24, and GA46, respectively. Moreover, AtGA2ox7 also produces small amounts of putative mono-unsaturated GA46 (compound b) from GA25, and 2α-hydroxylates C19-GA9 to GA40 similar to AtGA2ox9 (Fig. 1, Supplemental Fig. S3, Supplemental Table S1).

AtGA2ox9 and AtGA2ox10 GA 2-oxidases are catabolic enzymes. Previous studies identified class-III GA2ox7 and GA2ox8 as catabolic enzymes (Schomburg et al., 2003). Similarly, the overexpression of AtGA2ox9 and, less severely, of AtGA2ox10 in Arabidopsis results in a phenotype (dwarfism, late flowering, small dark green rosette leaves) that is typical for GA-deficient plants (Fig. 2A, Supplemental Fig. S4), indicating GA-catabolic function for both enzymes in planta. Consistently, endogenous GA110 levels in the AtGA2ox9 overexpressors are extremely elevated whereas levels of almost all other GAs of the pathway are reduced, including bioactive GA4 (Supplemental Table S3). Moreover, endogenous GA25 levels are also elevated, indicating GA 20-oxidase function of AtGA2ox9 in planta confirming the activity found of the respective recombinant enzyme (see above).

AtGA2ox9 and AtGA2ox10 are expressed during seedling and seed development, respectively. To evaluate the role of the identified class-III GA 2-oxidases in plant development, we analyzed their expression levels at different developmental stages of Arabidopsis by reverse transcription quantitative PCR (RT-qPCR; Fig. 2B). Expression analysis revealed that AtGA2ox9 is expressed throughout the Arabidopsis lifecycle with the highest transcript levels observed in mature seeds and in shoots and roots of 10-day-old seedlings. AtGA2ox10 was expressed mostly in siliques (Fig. 2B) and in developing seeds according to the Arabidopsis transcriptome analysis proposed by Klepikova et al. (2016). These results suggest that AtGA2ox9 and AtGA2ox10 play a
role in seedling and seed development, respectively. Moreover, transcript profiles of AtGA2ox7,
AtGA2ox8, and AtGA2ox9 were similar in 10-day-old seedlings and flowers of 35-day-old plants, and AtGA2ox7 expression levels were similar to those of AtGA2ox9 in mature seeds, suggesting functional redundancies.

AtGA2ox10 regulates fertility. Homozygous lines were obtained for two independent alleles of loss-of-function mutants of AtGA2ox9 (Atga2ox9-1 and Atga2ox9-2) and AtGA2ox10 (Atga2ox10-1 and Atga2ox10-2) by genotype PCR screening (Supplemental Table S4). To validate the roles of AtGA2ox9 and AtGA2ox10 in plant development, we analyzed the phenotypes of Atga2ox9-1 and Atga2ox10-1 loss-of-function mutants in comparison to wild-type (Col-0) Arabidopsis plants (Fig. 3A-C). In 7-day-old seedlings, hypocotyls of both Atga2ox9-1 and Atga2ox10-1 mutants were significantly longer when compared to wild-type plants (Fig. 3A). However, no differences were observed in root length and radicle emergence between 7-day-old mutant and wild-type plants. The similar development of 5-week-old Atga2ox9-1 mutant and wild-type plants suggests functional redundancy among GA 2-oxidase family members in adult plants, as predicted by the expression studies (Figs. 2B, 3B, C). However, Atga2ox10-1 knockout mutants flowered slightly earlier and developed more side branches on the main bolt compared to wild-type plants (Fig. 3B). As suggested by transcript analysis, AtGA2ox10 plays an important role in reproductive development. Loss-of-function mutants of the two independent alleles of AtGA2ox10, namely Atga2ox10-1 and Atga2ox10-2, displayed an increased number of seeds per silique compared to wild-type plants (Fig. 3B, Supplemental Table S5). To verify the GA status, we analyzed endogenous GA-levels in siliques of Atga2ox10-1 mutant and wild-type plants (Fig. 3D). Levels of GA12, the precursor of C19-GAs, significantly increased in the mutant plants, as expected, indicating a lack of AtGA2ox10 activity. Consequently, the Atga2ox10-1 mutants had slightly elevated bioactive C19-GA4 levels compared to wild-type plants, which we propose is responsible for the mutant phenotype observed, including elevated seed production (Fig. 3A-C).

AtGA2ox9 contributes to freezing tolerance. C-repeat/DREB binding factors (CBFs) are transcription factors known to be involved in cold adaptation of plants (Shi et al., 2018). Transcript levels of the C-repeat/DREB binding factors CBF1, CBF2, and CBF3, as expected, increased after the onset of the cold treatment, followed by class-I/II 2-oxidases AtGA2ox1 and AtGA2ox6, and that the highest transcript levels of the class-III GA 2-oxidases AtGA2ox8 and, particularly, AtGA2ox9 were observed much later after 12 hours of cold treatment (Fig. 4A and
Supplemental Table S6. For the other two members of the GA 2-oxidase family (AtGA2ox7 and AtGA2ox10), transcript levels did not change or were much less affected by the cold treatment.

We further investigated the impact of cold treatment on the GA-status of wild-type and

Atga2ox9-1 knock-down plants (Fig. 4B). At normal temperature, endogenous GA-levels of Col-0 and Atga2ox9-1 mutant plants were similar. Consequently, wild-type and Atga2ox9-1 mutant plants developed similarly. In wild-type plants, after chilling, catabolic GA34 and GA110 levels increased, indicating the action of class-I/II and class-III GA 2-oxidases, respectively, and bioactive GA4-levels remained low. However, in cold-treated Atga2ox9-1 mutant plants, only catabolic GA34 and GA51 increased indicating class-I/II GA 2-oxidases activity as proposed previously (Achard et al., 2008), and GA110-levels did not change. Consequently, precursor GA12, GA15, GA24, and bioactive GA4 levels accumulated in the Atga2ox9-1 mutant compared to wild-type plants (Fig. 4B). We further tested the role of AtGA2ox9 for freezing tolerance using two independent alleles of knock-down mutants, Atga2ox9-1 and Atga2ox9-2, and wild-type plants.
The survival rate was reduced in both mutants compared to wild-type plants after freezing (Fig. 4C, Supplemental Table S7). We conclude that, in Arabidopsis, AtGA2ox9 contributes to acquired cold stress tolerance.
Here we present the identification and characterization of two GA 2-oxidases, namely AtGA2ox9 and AtGA2ox10. Both are related to the previously described class-III GA 2-oxidases; however, they form a new cluster of phylogenetic relationships (Supplemental Fig. S1; Schomburg et al., 2003). Their amino acid sequences contain conserved motifs typical for 2-oxoglutarate-dependent dioxygenases, and residues involved in iron- and in 2-oxoglutarate-binding (Supplemental Fig. S2; Porco et al., 2016). Previously, it was proposed that a unique peptide sequence, consisting of 29 amino acids, might be responsible for the specific catalytic properties of class-III GA 2-oxidases (Schomburg et al., 2003). However, both AtGA2ox9 and AtGA2ox10 contain 10 extra amino acids within this peptide region, raising the question of whether their enzymatic properties are similar to those of the known class-III GA 2-oxidases (Supplemental Fig. S2). Indeed, AtGA2ox9 and AtGA2ox10 recombinant enzymes were found to 2ß-hydroxylate the C₂₀-GA precursor GA₁₂ to GA₁₁₀, which is a typical reaction for this class of GA 2-oxidases (Table 1; Schomburg et al., 2003; Lange and Pimenta Lange 2020). In addition, AtGA2ox9 and AtGA2ox10 harbor enzymatic properties previously not associated with GA 2-oxidases. AtGA2ox9 hydroxylates the C₁₉-GAs GA₉ and GA₄ at 2α-position to form GA₄₀ and GA₄₇, respectively. These 2α-hydroxylated GAs were found previously in the fungus *Fusarium fujikuroi*, but are not commonly identified in higher plants (MacMillan 2002). Moreover, the new AtGA2ox9 and AtGA2ox10 oxidize GA₁₅ at C-20 position. Such an enzymatic activity was first discovered to occur in cell extracts from spinach (*Spinacia oleracea*) leaves (Gilmour et al., 1986) and barley (*Hordeum vulgare*) seedlings (Grosselindemann et al., 1992). Recently, we identified GA 20-oxidases from Arabidopsis and cucurbits (*Cucurbitaceae*) that catalyze this reaction as well (Lange and Pimenta Lange, 2020). The two identified Arabidopsis GA 2-oxidases also oxidize GA₂₄, the next step in the GA biosynthetic pathway, to form either the C₁₉-GA GA₉ (AtGA2ox10) or the C₂₀ tricarboxylic acid GA₂₅ (AtGA2ox9). The formation of GA₉ is common for anabolic GA 20-oxidases, but production of the tricarboxylic acid, as in GA₂₅, is unusual and has been previously found for the catabolic GA 20-oxidases from pumpkin (*Cucurbita maxima*; CmGA20ox1 and CmGA20ox2) and, recently, from Arabidopsis (AtGA20ox5, Lange and Pimenta Lange, 2020). Such GA tricarboxylic acids are also endogenous to Arabidopsis and other plant species (Talon et al., 1990; MacMillan, 2002) and class-III GA 2-oxidases like AtGA2ox9 might contribute to their biosynthesis. Forming GA...
tricarboxylic acids might be another strategy that plants develop to inactivate GAs in addition to
the classical catabolic pathways (Magome and Kamiya, 2016): overexpression of the pumpkin
CmGA20ox1 leads to severe dwarfism in several plant species, including Arabidopsis, lettuce
(Lactuca sativa), and Solanum dulcamara (Curtis et al., 2000; Niki et al., 2001; Radi et al.,
2006). Other products of AtGA2ox9 enzyme activity include putative dehydrogenated GAs
(compound a and b, Supplemental Table S1), similar activities of which have been suggested for
pumpkin CmGA20ox1 since transgenic Solanum plants overexpressing this enzyme produce
putative dehydro GAs (Curtis et al., 2000).

Magome et al. (2008) also reported that the class-III GA 2-oxidase AtGA2ox7 also
converts C₂₀-GA precursors, including GA₁₅ and GA₂₄. By reinvestigating the AtGA2ox7
catalytic properties, we identified the products of GA₁₅ and GA₂₄ incubations as 2ß-hydroxy-
GA₁₅ and 2ß-hydroxy-GA₂₄, respectively. Moreover, both AtGA2ox7 and AtGA2ox9 also 2ß-
hydroxylate C₂₀-GA₂₅ to GA₄₆, a catalytic property previously identified for a bifunctional
pumpkin GA 3-oxidase, CmGA3ox1 (Lange et al., 1997). Similar to AtGA2ox9, we identified
2α-hydroxylase and putative desaturase activities with AtGA2ox7. These previously unassociated
activities of class-III GA 2-oxidases might have misled the interpretation of results previously
obtained for salt- or touch-stressed plants in which AtGA2ox7 is strongly expressed, without a
corresponding increase of endogenous 2ß-hydroxylated GAs (Magome et al., 2008; Pimenta
Lange and Lange, 2015). These activities might also add to the catabolic properties of
multifunctional class-III GA 2-oxidases: e.g. tobacco (Nicotiana tabacum) plants overexpressing
AtGA2ox7 display a more severe dwarf phenotype compared to those overexpressing AtGA2ox8
(an enzyme without GA 2α-hydroxylase function; Schomburg et al., 2003).

Taken together, Arabidopsis class-III GA 2-oxidases have heterogeneous enzymatic
properties. AtGA2ox8 is the only monofunctional member of the class-III GA 2-oxidases in
Arabidopsis (Fig. 1A). The other three members show broader substrate specificities: AtGA2ox7
and AtGA2ox9 also perform 2α-hydroxylation of C₁₉-GAs, whereas AtGA2ox9 and AtGA2ox10
oxidize the C₄–C₁₀ δ-lactone of GA₁₅ leading to GA₂₄ and produce tricarboxylic C₂₀-GAs
(GA₂₅, GA₄₆, compound b) and C₁₉-GA₉, respectively.

Class-III GA 2-oxidases mediate the integration of endogenous and exogenous signals
into the plant life cycle program. Although the complexity of the physiological and biochemical
responses after stress treatment often makes it difficult to analyze the role of GAs based on
phenotypic criteria (Lantzouni et al., 2020), in this study, our phenotypical analyses support the
biochemical data. We found that AtGA2ox9 (together with AtGA2ox7) is highly expressed in 10-
day-old shoots and in mature seeds. AtGA2ox10 is highly expressed in siliques (Fig. 2B) and
more specifically in developing seeds, as has been proposed by Klepikova et al. (2016),
suggesting that these class-III GA 2-oxidases play a role in GA homeostasis in these organs.
Indeed, the hypocotyls of 7-day-old Atga2ox9-1 and in the Atga2ox10-1 loss-of-function mutants
are longer, indicating a role of class-III GA 2-oxidases in early seedling growth, similar to class-
I/II GA 2-oxidases (Rieu et al., 2008). Interestingly, the height of 5-week-old plants was seen to
be reduced in the Atga2ox10-1 mutant with a significant increase in the number of branches on
the main bolt (Fig. 3B).

For efficient pollination, coordinated growth of pistil and stamen filaments is necessary
and this is regulated by gibberellins (Griffith et al., 2006; Pimenta Lange et al., 2012; Pimenta
Lange and Lange, 2016). Knock-out mutants of two C20-GA2oxs in rice resulted in a low seed-set
rate, indicating a potential role of class-III GA 2-oxidases in fertility and seed development (Chen
et al., 2019). Both elevated GA-levels and GA-deficiency in plants results in a loss of fertility
(Radi et al., 2006; Rieu et al., 2008; Jacobsen and Olszewski, 1993). These findings indicate that
to improve fertility it is necessary to achieve optimal GA levels. We found that fertility increased
significantly in the Atga2ox10-1 mutant probably due to a moderate modulation of the GA
hormone pool, as we observed previously for Arabidopsis plants overexpressing pumpkin
CmGA7ox or CmGA3ox1 (Radi et al., 2006).

Cold acclimatization is an important adaption of plants to low temperatures and is
regulated by C-repeat/DREB binding factors (CBFs) transcription factors (Shi et al., 2018).
Achard et al. (2008) demonstrated that DELLAs contribute to CBF1-induced cold acclimatization
and freezing tolerance. Recent studies demonstrated the cold-induced expression of At5g58660
(AtGA2ox9) gene as part of the CBF-regulon (Zhao et al., 2016). Moreover, Lantzouni et al.
(2020) showed that AtGA2ox8 expression increases after cold treatment, although gene responses
were followed for only 4 h after the onset of the cold-stress treatment. Here we found that the
class-I/II GA 2-oxidases (AtGA2ox1 and AtGA2ox6) respond earlier upon cold stress (3–6 h after
the treatment) compared to class-III GA 2-oxidases (AtGA2ox8 and AtGA2ox9, 12 h after
treatment), suggesting different regulation and function of class-I/II and class-III GA 2-oxidases upon cold stress.

The endogenous GA levels we observed in response to freezing stress support such interplay of class-I/II and class-III GA-oxidases. In wild-type plants, GA products of class-I/II (GA<sub>34</sub>) GA 2-oxidases first increased followed by those of class-III (GA<sub>110</sub>). As a result, bioactive GA<sub>4</sub> levels remained low. In the *Atga2ox9-1* mutant plants, early catabolism by class-I/II GA-oxidases occurred and the products (GA<sub>51</sub>, GA<sub>34</sub>) accumulated after chilling stress. However, the missing late activity of catabolic AtGA2ox9 led to an accumulation of GA precursors and of bioactive GA<sub>4</sub> levels in the mutant plant. This elevated biosynthesis of GA<sub>4</sub> is likely due to cold stress induction of GA 20-oxidase and GA 3-oxidase expression, as has been observed previously (Achard et al., 2008). Given that wild-type plants are more freezing tolerant compared to the *Atga2ox9* mutants, we conclude that AtGA2ox9 activity maintains low bioactive GA<sub>4</sub> levels, which is essential for survival under such unfavorable growth conditions.

We conclude that the identified class-III GA 2-oxidases AtGA2ox9 and AtGA2ox10 are catabolic enzymes playing pivotal roles in plant stress responses and developmental processes of high economic and ecologic relevance. In other plant species, class-III GA 2-oxidases with similar functions to AtGA2ox9 and AtGA2ox10 may exist, but enzymatic characterization of these putative family members remains to be performed.

Fine-tuning of bioactive GAs is particularly important for plant fertility and AtGA2ox10 substantially regulates this process. AtGA2ox9 is cold regulated and contributes to acquiring freezing tolerance, a task previously attributed to some members of class-I/II GA 2-oxidases. It is now tempting to study the role of class-I/II and class-III GA 2-oxidases in plant development and precise adaption to stresses in a spatiotemporal manner.
METHODS

**Plant Materials and Growth Conditions.** Arabidopsis Columbia (Col-0) was used as the wild type. The following mutant lines were obtained from the European Arabidopsis Stock Centre (NASC) and are all in the Col-0 background: At5g58660 (Salk_030540, Atga2ox9-1; Salk_030539, Atga2ox9-2); At3g47190 (Salk_053952C, Atga2ox10-1; Salk_071088, Atga2ox10-2). Homozygous lines were obtained by genotype PCR screening (described below). For soil-grown plants, seeds were sown on soil–vermiculite [2:1 v/v, 156 g per pot, 92% field capacity (FC)] in 24-pot trays, with 12 pots containing Col-0 and 12 pots the respective mutant line. To minimize positional effects within the growth chambers, pots were placed in a checkerboard-like pattern on the tray, and trays were rotated 180° every other day. Soil humidity was kept between 46% and 76% FC. Plants were grown in growth chambers with 70% humidity and a 16-h light/8-h dark cycle at 22°C/20°C, respectively (Atga2ox9-1 and Atga2ox10-1 at photon fluence rate of 200 µmol m⁻² s⁻¹, Osram Cool-White Fluorescent lamps; Atga2ox9-2 and Atga2ox10-2 at photon fluence rate of 300 µmol m⁻² s⁻¹, Osram Powerstar HQI-T 400W/D daylight lamps). For radicle-emergence, hypocotyl, and root-length measurements, seeds were sown on ½ MS agar square Petri dishes, stratified at 4°C for 3 days and then placed vertically in a controlled environment chamber (16-h light (photon fluence 200 µmol m⁻² s⁻¹)/8-h dark cycle at 22°C/20°C, respectively). Radicle emergence was monitored with the help of binoculars of at least 50 seedlings per genotype. Seven days after sowing the seeds, hypocotyl and root length were measured from at least 15 seedlings per genotype. For phenotypic analysis of adult plants, the flowering time was scored when the first flower bud was visible at the apex. The number of siliques and plant height (main inflorescence) was scored on 5-week-old plants using at least 15 plants for the measurements. For each genotype, siliques length of at least 90 siliques was measured and the number of seeds counted in at least 20 siliques per genotype.

**Plant cold/freezing assays.** Prior to stress treatment, pots containing 3–5 two-week-old plants per pot were adjusted to 61% FC. For cold treatment, plants were incubated for up to 26 h at 4°C maintaining the photoperiod described above. Plant freezing assays were performed as described by Eremina et al. (2016) with modifications. For freezing assays, plants were incubated in the dark in a controlled temperature chamber (FitoClima 600) for 30 min at 4°C. The temperature was then decreased to 0°C at a rate of 4°C per h, and further to −5°C at a rate of 2°C per h. This final temperature was maintained for 10 h (Atga2ox9-1) or 6 h (Atga2ox9-2) before the...
temperature was increased again to 4°C at a rate of 2.25°C per h. The plants were kept for one day at 4°C in a 16-h light (photon fluence 200 μmol m⁻² s⁻¹)/8-h dark photoperiod before they were returned to the standard growth conditions as described above. Survival rates were determined 2 weeks (Atga2ox9-1) or 1 week (Atga2ox9-2) after treatment, with only those plants able to develop new leaves counted as survivors.

**Genotype PCR.** Genomic DNA of the Arabidopsis mutant lines was isolated by the CTAB method using 20 mg of plant material. Genotype PCR reactions (20 μL) were performed using 0.5 μg of DNA as a template together with gene- and T-DNA-specific primers. Primers used are listed in Supplemental Table S8.

**Cloning of GA 2-oxidases from Arabidopsis.** In a phylogenetic analysis, Porco et al. (2016) identified two putative 2-oxoglutarate dependent dioxygenases, At5g58660 and At3g47190, in the Arabidopsis genome that are closely related to the previously identified class-III GA 2-oxidases AtGA2ox7 and AtGA2ox8. To investigate their enzymatic activities, we first cloned the respective cDNA molecules of putative or known GA 2-oxidases into the pET101/D-TOPO expression vector. Total RNA (100 ng) from different Arabidopsis tissues (Supplemental Table S9) was isolated as described elsewhere (Pimenta Lange and Lange 2015) and was used in 10 μL reverse transcription reactions, to produce cDNA molecules using the PCRBIO cDNA synthesis kit (BIOSYSTEMS), according to the manufacturer’s indications. The cDNA molecules were used as templates in 10 μL PCR reactions containing 2x Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific) and sequence-specific primers designed according to the CDS of the Arabidopsis putative or known GA 2-oxidases. PCR conditions and length of the expected PCR products are listed in Supplemental Table S9. After re-amplification, PCR products were purified by agarose gel electrophoresis (GeneJet TM Gel extraction kit, Thermo Fisher Scientific) and cloned into pET101/D-TOPO expression kit (Invitrogen) following the manufacturer’s instructions. Positive clones were identified by PCR and respective plasmid DNAs sequenced on both strands.

**Heterologous Expression of Recombinant GA-oxidases.** Plasmid DNA of the cloned AtGA2ox7, AtGA2ox8, At5g58660 (AtGA2ox9), and At3g47190 (AtGA2ox10) was used to transform BL21 Star™ E. coli (Invitrogen), according to the manufacturer's instructions.
Production of recombinant GA-oxidases and protein induction was as described previously (Lange et al., 2005).

**Enzyme Assays and Analysis of Incubation Products.** 14C-labeled GAs were prepared as described elsewhere (Lange et al., 2005). 17-14C-labeled GA_{12} was a gift from Professor L. Mander, Canberra, Australia. Preparations of *E. coli* cell lysates (70 µL) were incubated with 2-oxoglutarate and ascorbate (100 mM each, final concentrations). FeSO_{4} (0.5 mM), catalase (1 mg/mL), and the 14C-labeled substrate (2 µL in methanol; 0.33 nmol for (1,7,12,18-14C_{4})-labelled GAs and 1 nmol for (17-14C)-labelled GAs) were added in a total volume of 100 µL and incubated for 4 h at 30°C. Variations in incubation conditions are specified for particular experiments. For the synthesis of 17,17-d2-labelled GA_{110} (giving a yield of 94%), incubation mixtures contained recombinant AtGA2ox7 (36 µL *E. coli* cell lysate prepared as described above), 17,17-d2-labelled GA_{12} (2 µg; from Professor L. Mander, Canberra, Australia), 2-oxoglutarate (4 mM), ascorbate (4 mM), FeSO_{4} (0.5 mM), and catalase (1 mg/mL) in a total volume of 50 µL were incubated for 16 h at 30°C. Analyses of incubation products by HPLC and GC–MS were done as described previously (Lange et al., 2005).

**Overexpression of CsGA2ox9 and CsGA2ox10 in Arabidopsis.** To express AtGA2ox9 and AtGA2ox10 in Arabidopsis, cDNA molecules were PCR amplified using Phusion High-Fidelity PCR Master Mix, as described above, with primers containing restriction sites (Supplemental Table S8) and cloned at the BamH1, EcoRI sites of a modified pBE2113 vector containing a strong promoter cassette and a translational enhancer (E12-35-Ω). *Agrobacterium tumefaciens*-mediated transformation of Arabidopsis and selection of transgenic lines was done as described previously (Pimenta Lange et al., 2020). After scoring at T3, two homozygous lines for each gene were taken to generate T4 homozygous plants. The lines were phenotypically similar (Fig. 2) and line 1 of each construct was utilized in the further studies (Supplemental Fig. S4).

**Gene expression analysis.** Total RNA extraction has been described elsewhere (Pimenta Lange and Lange 2015). First-strand cDNA synthesis was done with 500 ng of DNaseI-treated total RNA using the SuperScript IV VILO MasterMix in a 5-µL reaction volume according to the manufacturer’s protocol (Thermo Fisher Scientific). The cDNA was diluted 10 times with water and used for 10 µL RT-qPCR reactions as described previously (Pimenta Lange and Lange...
2015), but with the addition of 5 µL of qPCRBIO SyGreen No-Rox mix (PCR Biosystems) in a
two-step cycling program according to the manufacturer’s protocol. The At2g28390 (SAND)
gene was used as the internal reference gene. The real-time PCR Miner program was used to
calculate the relative expression of each gene (http://www.miner.ewindup.info/) as the average of
two technical qPCR replicates. Technical replicates with a difference from the mean Ct ≥ 0.5
were excluded. All experiments were done with at least three biological replicates. The samples
were tested for the absence of genomic DNA by qPCR using RNA as template. Primers used for
qPCR are listed in Supplemental Table S8.

**Analysis of endogenous gibberellins.** For quantitative analysis of endogenous GAs, 0.5 g fresh
weight of frozen, pulverized plant tissues were spiked with 17,17-d2-GA standards (between 0.4
and 6 ng, according to the expected amounts of endogenous GAs; from Professor Lew Mander,
Canberra, Australia), and 80% (v/v) methanol-water (1.5 mL) was added. The samples were
stored at –20°C for 16 h and centrifuged at 4,000 g for 5 min. Extraction and solvent partitioning
were performed as described elsewhere (Lange et al., 2005). Samples were dissolved in methanol
(2 mL) and after loading onto an ion-exchange column (BondElut DEA, Varian), the column was
washed with methanol (6 mL) and the GAs were eluted with methanol containing 1% (v/v) acetic
acid (6 mL). The samples were dried and redissolved in methanol (100 µL) and water (2 mL)
containing 1% (v/v) acetic acid. After loading the samples onto a C₁₈-cartridge (Waters), the
cartridge was washed with water (2x 10 mL) containing 1% (v/v) acetic acid. GAs were eluted
from the cartridge with 80% methanol-water containing 1% (v/v) acetic acid. The eluates were
then dried, derivatized, and analyzed by gas chromatography-mass spectrometry (Lange et al.,
2005). The ions monitored for quantification of endogenous GAs were 300 and 302 (GA₁₂), 298
and 300 (GA₁₁₀), 239 and 241 (GA₁₅), 314 and 316 (GA₂₄), 312 and 314 (GA₂₅), 298 and 300
(GA₉), 268 and 270 (GA₅₁), 284 and 286 (GA₄), and 506 and 508 (GA₃₄).

**Statistical Analysis.** For phenotypic characterization experiments, statistical analysis was
performed using Student’s t-test ($P < 0.01$). For GA levels and RT-qPCR data, statistical analysis
was performed using Student’s t-test ($P < 0.05$).

**Accession Numbers.** Sequence data from this article can be found in the GenBank/EMBL data
libraries under accession numbers: LT827066 (AtGA₂₀x₉) and LT827067 (AtGA₂₀x₁₀).

**SUPPLEMENTAL DATA**
Supplemental Figure S1. Phylogenetic relationships of the GA 2-oxidase family from Arabidopsis (At), cucumber (Cs), and rice (Os).

Supplemental Figure S2. Protein sequences alignment between the predicted AtGA2ox9 and AtGA2ox10 and the known Arabidopsis class-III GA 2-oxidases.

Supplemental Figure S3. Catalytic properties of recombinant class-III GA 2-oxidases AtGA2ox7 and AtGA2ox8.

Supplemental Figure S4. Phenotypic analysis of soil grown 14-day-old AtGA2ox9 and AtGA2ox10 overexpressors.

Supplemental Table S1. Mass spectra and Kovats retention indices (KRI) of products of the methyl ester trimethylsilyl ether derivatives from incubations of selected GAs with cell lysates from Escherichia coli expressing recombinant GA 2-oxidases from Arabidopsis, AtGA2ox7, AtGA2ox8, AtGA2ox9, and AtGA2ox10.

Supplemental Table S2. Metabolism of 14C-labelled GA substrates incubated for 4 h with different volume of cell lysates prepared from E. coli expressing recombinant AtGA2ox9.

Supplemental Table S3. Endogenous GA levels in 23-d-old soil grown Col-0 and 35S::AtGA2ox9, line 1 plants.

Supplemental Table S4. Characterization of knock-down and know-out lines for Atga2ox9 and Atga2ox10, respectively.

Supplemental Table S5. Number of siliques per plant and seeds per silique of Atga2ox10-2 knock-down mutant line in comparison to wildtype Col-0.

Supplemental Table S6. Transcript levels of Arabidopsis CBF1, CBF2, CBF3, and selected class-I/II GA 2-oxidase encoding genes.

Supplemental Table S7. Survival rate after freezing of Atga2ox9-2 knock-down mutant compared to Col-0.

Supplemental Table S8. List of primers used for overexpression in Arabidopsis, genotype and RT-qPCR.
Supplemental Table S9. Plant tissues and PCR conditions used for cloning the GA 2-oxidases.

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FIGURE LEGENDS

Fig. 1. Enzymatic properties of class-III GA 2-oxidases in Arabidopsis. A. Scheme of the reactions catalyzed by the two identified multifunctional enzymes AtGA2ox9 (At5g58660) and AtGA2ox10 (At3g47190), which are illustrated in bold characters. C$_{19}$-GAs are highlighted by a blue background, whereas all others are C$_{20}$-GAs. B. HPLC-radiochromatograms of products from incubations of recombinant AtGA2ox9 and AtGA2ox10 with the C$_{20}$-GAs $^{14}$C-GA$_{12}$, -GA$_{15}$, -GA$_{24}$, and -GA$_{25}$, and the C$_{19}$-GAs $^{14}$C-GA$_{9}$ and -GA$_{4}$ as the substrates. Radiochromatograms with C$_{19}$-GAs are in blue. Products were identified by GC-MS (Supplemental Table S1). Compound a, putative didehydro-GA$_{110}$; compound b, putative didehydro-GA$_{46}$.

Fig. 2. Catabolic function and sites of expression of class-III GA-2oxidases. A. Phenotype of transgenic plants overexpressing catabolic AtGA2ox9 and AtGA2ox10. Representative 32-day-old plants transferred to the soil after 18 days in Murashige and Skoog medium, from left to right: Col-0 and overexpressors (OE) two lines of AtGA2ox9OE and two lines of AtGA2ox10OE; Scale bar, 5 cm. B. Catabolic class-III GA2ox9 and GA2ox10 are expressed during seedling and seed development, respectively. Transcript levels of GA2ox7, GA2ox8, GA2ox9, and GA2ox10 genes were determined in different tissues by RT-qPCR (relative to SAND) during Arabidopsis development. Plotted data are means of three biological replicates ± SE.
Fig. 3. Analysis of AtGA2ox9 and AtGA2ox10 loss-of-function mutant plants. Phenotypic analysis of Atga2ox9-1 and Atga2ox10-1 mutants in comparison to wild type Col-0 A. Plate-grown 7-day-old seedlings B. Soil-grown 35-day-old plants. Plotted data are means of ≥15 plants, ≥90 siliques, and seeds from 20 siliques per genotype ± SE. C. Representative 35-day-old plants; (from left to right) Col-0, Atga2ox9-1, and Atga2ox10-1. D. Endogenous GA levels (means ± SE) in siliques of 35-day-old Col-0 and Atga2ox10-1 plants. Shown are means of three biological replicates (in ng g⁻¹ fresh weight). Statistical differences relative to Col-0 were determined by Student’s t-tests; * for p < 0.05, ** for p < 0.01; Scale bar, 5 cm.

Fig. 4. AtGA2ox9 contributes to freezing tolerance in Arabidopsis. A. Cold regulation of the Arabidopsis class-III GA 2-oxidase genes. Transcript levels of GA2ox7, GA2ox8, GA2ox9, and GA2ox10 genes were determined by RT-qPCR (relative to SAND) in Arabidopsis 2-week-old plants subjected to cold treatment (4°C) for different times as indicated or kept at 22°C (control). Data plotted are means ± SE of three biological replicates. B. Endogenous GA levels (means ± SE) in 14-day-old Col-0 and Atga2ox9-1 seedlings without or with cold treatment (12 h at 4°C). Shown are means of three biological replicates (in ng g⁻¹ fresh weight). In samples marked n.d. endogenous GA was not detected, but the internal standard was recovered. C. 14-day-old Col-0 and Atga2ox9-1 plants were exposed for 10 h to −5°C as described in Material and methods and survival was scored after 14-days of recovery; Top, representative plants after freezing treatment of Col-0 (two pots containing five plants each on the left) and Atga2ox9-1 (two pots containing five plants each on the right); Bottom, quantification of the results, data plotted are means of four biological replicates (n=36 for each replicate) ± SE. Statistical differences relative to Col-0 were determined by Student’s t-tests; * for p < 0.05, ** for p < 0.01; Scale bar, 5 cm.
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