USING THE REGULATION OF *GA2-OXIDASE* AS A MODEL TO UNDERSTAND GIBBERELLIN'S ROLES IN APPLE AND *ARABIDOPSIS THALIANA*

By

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ABSTRACT

USING THE REGULATION OF *GA2-OXIDASE* AS A MODEL TO UNDERSTAND GIBBERELLIN'S ROLES IN APPLE AND *ARABIDOPSIS THALIANA*

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In the model plant *Arabidopsis thaliana* (Arabidopsis), where the gibberellin (GA) signaling pathway has been best studied, GAs promote flowering. However, in many woody perennial plants such as apple (*Malus × domestica* Borkh.), GAs generally have a repressive role in flowering. The genetic and molecular mechanisms of GA-mediated flowering repression in apple and other plants remain unknown. In this study, we carried out transcriptional profiling of apple shoot apices during the anticipated period of floral induction and examined how gene regulation is influenced by exogenous GAs. GA treatment resulted in downregulation of a diversity of genes participating in GA biosynthesis, transport and reception, and strong upregulation of the GA catabolic *GA2-ox (GA2-oxidase*) genes, suggesting GA homeostasis is mediated at multiple levels in these tissues. We also observed rapid and strong upregulation of both of two copies of a gene previously observed to inhibit flowering in apple, *MdTFL1* (*TERMINAL FLOWER 1*), offering a potential explanation for the flowering-inhibitory effects of GA in apple. These results provide a context for investigating factors that may transduce the GA signal in apple and contribute to a preliminary genetic framework for the repression of flowering by GAs in a woody perennial plant.

GA2ox expression and the enzyme activity determines concentrations of specific GAs in the right structure at the right time and contributes to enabling GAs to execute their function precisely. In return, their expression is under tight regulation by GA. Taking

advantage of this tight link between *GA2ox* and GA activity, we aimed to develop regulation of *GA2ox* as a model to evaluate GA activity and function in both apple and Arabidopsis with a focus on floral induction. Through analysis of high-quality apple genomes and our transcriptional datasets, we identified 16 canonical *GA2ox*-like genes in the apple genome, representing eight pairs of homoeologous genes. By analyzing their spatial and developmental expression, we found that at least half of the genes were expressed in all the structures studied. In addition, individual members of homoeologous pairs displayed both overlapping and distinct expression patterns, suggesting function redundance and divergency. Finally, we found that two pairs of homoeologous genes (*MdGA2ox1A*, *-1B*, *-2A* and *-2B*) may be responsible for regulating GA activity in the shoot apex during floral induction. These findings provide biological and evolutionary insights into GA biology in apple, particularly in floral induction. In Arabidopsis, where *GA2ox* has been relatively well studied, we developed a genetic tool kit comprising both CRISPR/Cas-induced, transgene-free single knockout mutants and transcriptional and translational GUS fusion lines for the seven canonical Arabidopsis *GA2ox* genes (*AtGA2ox1-AtGA2ox8*). Mutations in the loss-of-function mutants have presumably disrupted function of both two functional domains of the GA2ox proteins. By comparing GUS activity in the GUS fusion lines, we found that the transcribed region is required for appropriate expression for several genes. This tool kit can be used for a wide spectrum of GA biology.

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Dedicated to Philip John Engelgau

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CHAPTER ONE

Literature Review

Introduction

Plants are “magical” organisms. They do not speak to express their feelings or needs; they do not physically run away like animals when being threatened. Being quiet and sessile, however, does not mean that they always have to take everything on passively. Plants have their own survival wisdom and strategies. They constantly make inner efforts that cannot easily be seen with the naked eye, to grow and to survive. Plant hormones are one of their secrets to surviving the ever-changing surroundings.

Plant hormones are small organic molecules that control all aspects of a plant life, from cell expansion and division to organ differentiation, from vegetative to reproductive growth, from bacterial and viral defense to abiotic stress defense. To grow and survive, plants need to adjust their growth and defense systems by regulating the timing and place of plant hormones in response to environmental cues. These hormones are produced and transported to their sites of action, perceived by specific receptors, and transduced to modulate gene transcription and protein interaction (Taiz et al. 2015).

Gibberellins (GAs) are a class of phytohormone that is widely present in vascular plants and several plant-associating fungi and bacteria (Hedden 2020). GAs have profound effects on numerous aspects of plant growth and development. A well-documented role of GA is to promote flowering in herbaceous rosette plants such as the model plant *Arabidopsis thaliana* (Arabidopsis) (Davies 2010). At the molecular level, GA promotes flowering mainly by upregulating the expression of a floral gene *LEAFY* (Blázquez et al. 1998; Eriksson et al. 2006; Yamaguchi et al. 2014). In contrast to its promotive role in flowering in annual plants, GA has a generally repressive role in flowering in perennial woody plants such as apple, citrus, peach, sour cherry, and

perennial rose (see below). However, little is known about the underlying genetic and molecular mechanisms.

Based on GA's repressive role in floral induction, a theory has been developed for alternate bearing. Alternate bearing is one of the production challenges in apple and many other tree fruits. As the name indicates, alternate bearing is a phenomenon where trees produce heavy and light crop loads in successive years (Monselise and Goldschmidt 1982; Krasniqi et al. 2017). In many apple cultivars, current-year fruits and buds destined for next-year fruits or shoots are developed on the same shoots, spur shoots, and fruit development coincides with floral induction. Seeds of the developing fruits produce a significant amount of bioactive GAs, which can be subsequently transported into the adjacent shoot apex (Luckwill et al. 1969; Ramírez et al. 2001). It is proposed that the seed-derived GAs repress floral induction, and thus lead to a low crop load the following year. However, there is currently no solid evidence supporting this hypothesis, hindering development of solutions to alternate bearing.

The goals of my dissertation research were: 1) to study the genetic and molecular mechanisms underlying GA's repressive role in flowering in one of the most cultivated tree fruits, apple (*Malus x domestica* Borkh.), by identifying the GA-responsive genes that link GA with flowering (Chapter 2); 2) to study apple GA biology with a focus on floral induction using the positive regulation of GA catabolic genes, *GA2-oxidase* (*GA2ox*) (Chapter 3); 3) to develop a *GA2ox* toolkit in the model plant Arabidopsis for mechanistic studies on GA biology (Chapter 4).

GA in Agricultural and Horticultural Crops

Since identification of the first gibberellin in a fungus causing lodging in rice in the 1930s, our understanding of GA function, metabolism and signaling in plants has been significantly advanced. This has been largely credited to genetic studies using GA-deficient and GA-insensitive mutants. A notable example is the high-yielding rice and wheat varieties that contributed to the Green Revolution between 1950 and the late 1960s. These varieties either contained mutations in the GA biosynthesis genes or GA signaling component genes, which led to GA deficiency and conferred their dwarf and high-yielding traits (Sakamoto and Matsuoka 2004).

As our understanding of GA continues to grow, GA has been widely used as a plant growth regulator in many aspects of agricultural management and production. A well-known example is the use in grapevine. In grapevine, GA is applied to combat bunch rot, as the application of GA can reduce cluster compactness and reduced compactness allows less berries to touch one another (Weaver et al. 1962). Another important use of GA is to promote fruit enlargement in seedless table grapes (Weaver 1958). As seeds are a major source of GA, seedless grapes tend to grow small due to the lack of internal GA. Use of GA has solved this problem and brought large, seedless grapes to our table.

Another significant use of GA in horticultural crops is demonstrated in sour cherry by Dr. Martin John Bukovac (Bukovac et al. 1986; Bukovac and Yuda 1991). Sour cherry yellows is a disease caused by prune dwarf virus, which is transmitted mainly through pollen (George and Davidson 1963, 1964). Leaves of infected trees turn yellow and eventually drop early. In addition, the viral infection leads to the formation of dormant buds and thus unproductive wood. As a result, it significantly reduces yield. Young sour cherry

trees tend to produce excessive flowers, which is favorable for the transmission of the virus. Dr. Bukovac's research group succeeded in combating the disease by simply spraying GA on infected young sour cherry trees. GA reduces return bloom in sour cherry and thus slows down the transmission of virus. GA also breaks the dormancy of blind wood, enhances growth of shoots or fruiting units, and eventually promotes cropping efficiency.

Apple is no exception to the use of GA. For example, in springtime some young fruits drop around May or June, which is called June drop. While June drop can be beneficial in such a way that it thins out small fruits and ensures the growth of healthy ones, a considerable number of fruits can drop under stresses such as drought, cold and shade. In this case, GA can be sprayed to enhance fruit set before the massive drop (Wertheim 1982), although an accurate estimate of the drop is usually tricky.

GA Metabolism and Signaling

GAs comprise a wide variety of diterpenoid compounds sharing a basic structure (Fig. 1). To date, 136 molecularly distinct GAs have been identified, and these have been designated as GA_1 - GA_{136}. Four of these - GA_1, GA_3, GA_4 and GA_7 - are known to have biological activity in flowering plants. The biosynthesis of gibberellins (GA) in higher plants involves a series of reactions catalyzed by several groups of enzymes in cellular compartments (Hedden and Thomas 2012). Briefly, GA biosynthesis can be summarized into three major steps (Fig. 2). In the first step, GGPP is cyclized by the consecutive action of two monofunctional diterpene cyclases, CPS (*ent*-copalyl diphosphate synthase) and KS (*ent*-kaurene synthase) to yield *ent*-kaurene in proplastids. In the second, *ent*-kaurene is converted to the first GA form, GA_{12}, through sequential oxidation by two cytochrome

P450 mono-oxygenases, plastid membrane-bound KO and endoplasmic reticulum-bound KAO. The last step is the conversion of C20-GA precursors into bioactive C19-GAs through succeeding oxidations catalyzed by two groups of 2-oxoglutarate–dependent dioxygenases (2-ODDs): C-20 is oxidized and removed from the C20-GA backbone by GA20ox (GA20-oxidases); 3β-hydroxylation by GA3ox (GA3-oxidases) converts non-bioactive C19-GAs into bioactive C19-GAs, including GA_1, GA_3, GA_4 and GA_7. It is believed that the third step, more specifically, C-20 oxidation by GA20ox, is the limiting step in GA biosynthesis (Huang et al. 1998; Coles et al. 1999; Fleet et al. 2003).

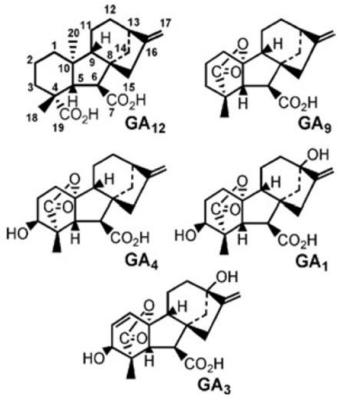

Figure 1. Structures of the simplest C20-GA (GA_{12}), C19-GA (GA_9) and the major bioactive GAs (GA_1, GA_3 and GA_4). Original figure is from Hedden and Thomas 2012.

An additional group of 2-ODD enzymes, GA2-oxidases (GA2ox), catalyze 2β-hydroxylation of both bioactive and inactive GAs, and represent a major mechanism for catabolism of GAs and the resetting of GA signaling (Rieu et al. 2008) (Fig. 2). In addition to this major pathway for GA inactivation, two GA2ox-independent inactivation pathways have been reported. One involves a cytochrome P450 monooxygenase, EUI (ELONGATED UPPERMOST INTERNODE). EUI targets non-13-hydroxylated GAs, in particular GA_9 and GA_4 to produce 16,17-$[OH]_2$-GAs, which have been found in rice, apple and many other plant species (Hedden et al. 1993; Zhu et al. 2006; Zhang et al. 2011),

suggesting that EUI-dependent GA inactivation might be a general mechanism in plants. However, it is not clear to what extent GA inactivation is dependent on this pathway in plant growth and development, compared with GA2ox catabolism. Another involves methylation of GAs via GAMT1 (Gibberellin Methyltransferase 1) and GAMT2, and analysis of loss-of-function mutants suggests their role in GA inactivation in seed development (Varbanova et al. 2007). Finally, bioactive GAs can be deactivated through conjugation to other small molecules such as glucose (Schneider and Schliemann 1994).

In all plants yet studied, proteins mediating GA biosynthesis and catabolism are encoded by multigene families. In Arabidopsis, there are five paralogous *GA20ox* genes (Hedden and Phillips 2000). Three of these, *GA20ox1*, *-2*, and *-3*, seem to be the dominant paralogs, because disruption of these genes has the greatest impact on plant development (Plackett et al. 2012). Four *GA3ox* genes have been identified and designated as *GA3ox1*, *-2*, *-3* and *-4* (Mitchum et al. 2006). Like GA20ox and GA3ox, GA2ox enzymes are also encoded by multiple genes. To date, nine GA2ox-encoding genes have been characterized in Arabidopsis, namely *GA2ox1*-*GA2ox10* with *GA2ox5* being a pseudogene (Thomas et al. 1999; Hedden and Phillips 2000; Schomburg et al. 2003; Lange et al. 2020). Gene encoding GA metabolic enzymes have been predicted to exist in seed plants, ferns and lycophytes (Hernández-García et al. 2021). Members of these families may have distinct functions based on expression patterns, expression levels, and/or enzymatic properties (see below).

The molecular basis of the GA signaling pathway comprises four elements: (1) bioactive GAs, (2) GID1 (GIBBERELLIN- INSENSITIVE DWARF 1) and related proteins, which act as GA receptors, (3) a class of transcriptional repressor proteins designated

DELLA, and (4) a set of transcription factors (TFs) that initiate changes in downstream GA response-associated genes (Hirano et al. 2008) (Fig. 3). The most convincing model of DELLA action is that DELLA proteins hijack TFs from activating the downstream genes by direct protein-protein interaction. In all examples studied so far, binding of GA to GID1-type receptors promotes interaction between GID1 and a DELLA protein, which then triggers targeting of the DELLA protein by the $SCF^{GID2/SLY1}$ complex (Ueguchi-Tanaka et al. 2007; Hirano et al. 2008). $SCF^{GID2/SLY1}$ is an E3 ligase that promotes ubiquitination of the targeted DELLA proteins, hence initiating their degradation by the 26S proteasome and the release of their target TFs (Sun 2010). Once freed from their bondage to DELLA proteins, TFs participate in regulating transcription at GA-responsive genes (Xu et al. 2014) (Fig. 3).

The *GID1* gene was first identified in rice, and the encoded GID1 protein is localized and active in both the cytoplasm and nucleus (Ueguchi-Tanaka et al. 2005). Three GID1-like GA receptors have been identified in Arabidopsis: GID1a, GID1b and GID1c (Nakajima et al. 2006). The corresponding genes are distinguished both by overall expression level and by developmental regulation. For example, *GID1a* is generally expressed at ~10-fold higher levels than *GID1b* or *GID1c*, and *GID1b* shows strong localized expression in the shoot apex (our unpublished analysis of data published by (Schmid et al. 2005).

Arabidopsis has five genes encoding DELLA proteins: *GAI (GIBBERELLIC ACID INSENSITIVE)*, *RGA (REPRESSOR OF GA1-3)*, *RGL1 (RGA-LIKE 1)*, *RGL2* and *RGL3* (Tyler et al. 2004). DELLA proteins are generally considered to be negatively acting components of GA signaling, as loss of DELLA function mainly results in upregulation of

GA-responsive genes and confers the phenotype of continuous GA treatment (Dill and Sun 2001; de Lucas et al. 2008; Feng et al. 2008). It is believed that DELLA proteins do not contain a DNA-binding domain, and thus their function is much dependent on interaction with other proteins, usually transcriptional factors. DELLA proteins have numerous interacting targets, and their targets can act on multiple GA-responsive genes. This vast web of regulation enables GA to affect a wide spectrum of growth and developmental processes. DELLA proteins are present in both vascular plants and bryophytes (Hernández-García et al. 2019), while GID1 is found functional only in vascular plants (Hirano et al. 2007; Yasumura et al. 2007). Thus, it is thought the GA perception module evolved following DELLA proteins (Hernández-García et al. 2021).

GA signaling also can employ the GAMYB subclass of R2R3-MYB TFs (Woodger et al. 2003). The founding member of this class, barley GAMYB, participates in GA-induced seed germination by binding to a *cis*-element within the promoter of an alpha-amylase gene to activate its expression (Gubler et al. 1995). GAMYB also mediates the role of GA in development of the anther (Murray et al. 2003; Aya et al. 2009). In Arabidopsis, the homologous *AtMYB33* is required to promote flowering (see below).

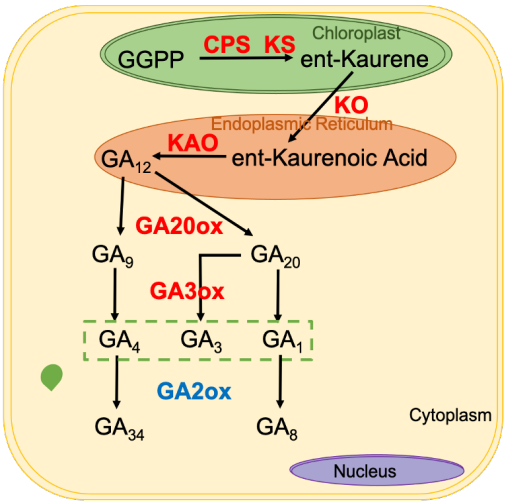

Figure 2. Gibberellin metabolism pathway. GA biosynthetic enzymes are highlighted in red and GA catabolic enzymes in blue. Bioactive GAs are highlighted in the green box with dashed lines.

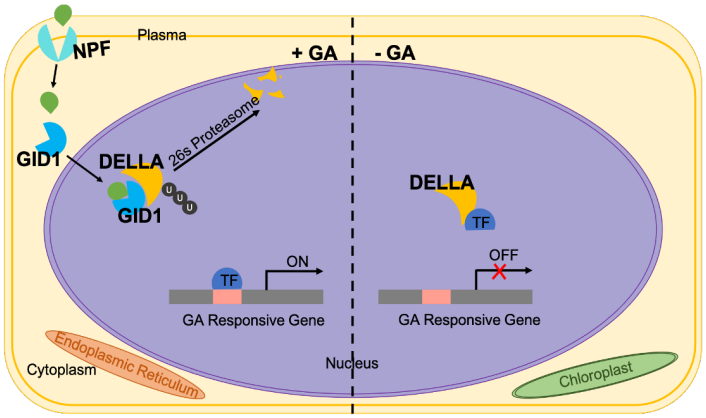

Figure 3. Gibberellin signaling pathway. This illustration is adapted from Xu et al. (2014). Bioactive GAs are depicted with the green water drop-shaped symbol. NPF is a GA influx transporter, GID1 represents GA receptor, and DELLAs are the integrative regulator in GA signaling pathway. *Cis*-regulatory elements of GA responsive genes are depicted with the pink box. Transcription factors that bind to these *cis* elements are shown in dark blue.

GA Homeostasis and Its Genetic Control

Bioactive GAs have been shown to both repress GA biosynthesis and promote GA deactivation. Repression of biosynthesis (feedback regulation) is mediated by decreased expression of the GA biosynthetic genes, *GA20ox* and *GA3ox*, whereas promotion of

deactivation (feedforward regulation) is mediated by increased expression of the GA deactivation genes, *GA2ox* (Fig. 4). Studies in Arabidopsis and pea have suggested that DELLA proteins participate in feedback and feedforward regulation, as GA biosynthetic genes were strongly down-regulated, whereas *GA2ox* genes were strongly upregulated in loss-of-function *della* mutants (Zentella et al. 2007; Weston et al. 2008). Exactly how DELLA proteins are involved in regulation of *GA2ox* genes is unknown. For feedforward regulation, the simplest model is that a DELLA protein interacts with a transcriptional activator of a *GA2ox* gene, directly at a *GA2ox* promotive regulatory element (Fig. 4). Modeling feedback regulation is less intuitive, as a DELLA protein would be expected to interact with a transcriptional repressor, or to act indirectly at an upstream gene that encodes a repressor of GA biosynthesis (Fig. 4).

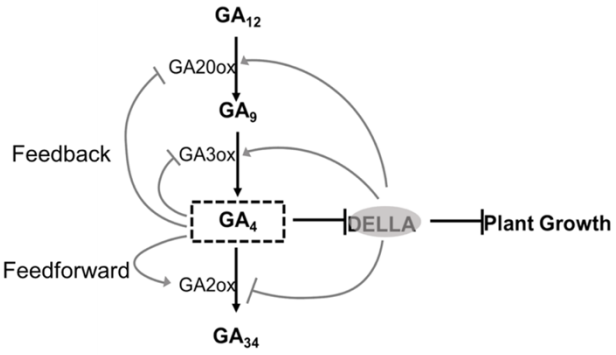

Figure 4. GA feedback and feedforward regulation of GA metabolic genes in Arabidopsis. Dotted box denotes a bioactive GA, GA_4; grey lines denote transcriptional regulation; grey oval box represents protein.

GA in Floral Induction

In Arabidopsis, promotion of flowering by GAs is indispensable under non-inductive short-day conditions, and this mechanism has been characterized as one of the five interacting flowering-promotive pathways (Blázquez et al. 1998). GAs ultimately upregulate expression of the flowering master regulator gene *LEAFY* (*LFY*). This is at

least in part dependent on binding of the GAMYB factor, AtMYB33, to a regulatory element within the *LFY* promoter (Gocal et al. 2001). *AtMYB33* mRNA accumulates in the shoot apex accompanying a transition to inductive long-day photoperiods, and in plants treated with exogenous GA_4. At least in some functional contexts, *AtMYB33* is subject to negative regulation by the microRNA miR159 (Tsuji et al. 2006). Ectopic overexpression of miR159 in transgenic Arabidopsis led to delayed flowering and reduced *LFY* levels (Achard et al. 2004). Whereas it could be hypothesized that miR159 may participate in, and negatively regulate, the GA flowering pathway, at least some evidence has shown that miR159 targets *AtMYB33* independently of GAs (Alonso-Peral et al. 2010). In addition, GA can upregulate expression of *LFY* through another floral promoter gene *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CO 1*) under short days (Moon et al. 2003; Lee and Lee 2010).

The promotion of flowering by GAs in Arabidopsis is in contrast to the generally repressive effects of GAs on flowering in perennial plants including apple, peach, citrus, sour cherry and perennial rose (Tromp 1982; Bukovac et al. 1986; Southwick et al. 1995; Bertelsen and Tustin 2002; Randoux et al. 2012; Goldberg-Moeller et al. 2013; Zhang et al. 2019). Domesticated apple has a biennial (two-year) and overlapping flowering cycle, such that flowers are initiated during one growing season on branches that are already in the reproductive phase, and complete development and bloom the subsequent season. Application of GAs to whole trees during the period of floral induction reduces bloom the following year (Guttridge 1962; Marcelle and Sironval 1963; Luckwill and Silva 1979; Bertelsen and Tustin 2002; Schmidt et al. 2009; Zhang et al. 2019). When carried out in

commercial operations, this approach optimizes fruit size and quality of several fruit trees (Greene 2000; Reig et al. 2006).

A potential mechanism for this phenomenon is that GA has an opposite effect on the apple counterparts of the interactive flowering promotive genes in Arabidopsis such as *FT* (*FLOWERING LOCUS T*), *LFY* and *SOC1*. Where studied, *FT* is a florigen-encoding gene that integrates flower-inducing signals from the photoperiod, autonomous and vernalization pathways. It is mainly expressed in leaf, the light-harvesting organ, and its product is translocated to the shoot apical meristem, where it interacts with another protein FD to activate the expression of downstream floral promoter genes including *AP1* (*APETALA 1*), *SOC1*, and *LFY* (Abe et al. 2005; Lee and Lee 2010). Two *FT*-like genes, *MdFT1* and *MdFT2*, have been characterized in the apple genome (Kotoda et al. 2010). Unlike their Arabidopsis homolog, *MdFT1* is predominantly expressed in the shoot apex of fruit-bearing shoots, while *MdFT2* mainly in reproductive organs (Kotoda et al. 2010). Apple plants overexpressing the Arabidopsis flowering gene *FT* had a continuous-flowering phenotype (Tanaka et al. 2014). Arabidopsis plants overexpressing either copy of *MdFT* flowered earlier than wild-type plants, and transgenic apple overexpressing *MdFT1* flowered in vitro 8-12 months after transformation, suggesting a conserved function in promoting flowering (Kotoda et al. 2010). Similarly, researchers identified two *LFY*-like genes in the apple genome, *MdAFL1* and *MdAFL2* (Wada et al. 2002). *MdAFL1* was predominantly expressed in the shoot apex during floral induction, whereas *MdAFL2* was expressed in multiple structures including the vegetative shoot apex, floral bud, and root. Ectopic overexpression of either gene in Arabidopsis led to an early-flowering phenotype (Wada et al. 2002). Interestingly, ectopic expression of the Arabidopsis *LFY*

in apple did not result in reduced juvenility stage but shortened internodes and plant height, resembling GA-deficient phenotypes (Flachowsky et al. 2010).

A recent study reported that GA may have dual opposite roles in flowering in Arabidopsis (Yamaguchi et al. 2014). It is proposed that prior to the floral transition, GA levels are elevated to promote termination of the vegetative stage and inflorescence branching through upregulation of *SPLs* and *LFY*. As GA levels subsequently decrease, DELLA proteins are free to activate expression of *AP1*, a downstream floral integrator, and thus the onset of flower formation either through direct interaction with *SPL9* or indirect interaction with *LFY*. This model could potentially explain GA's opposite effect on flowering in apple. In apple, fruit development coincides with floral induction. Seed-derived GAs can be transported to and accumulated in the adjacent shoot apex (Ramírez et al. 2001), where high levels of GA may be advantageous for termination of vegetative stage but disadvantageous for floral formation. This model is largely dependent on regulation of *LFY* either directly or indirectly by GA.

Alternatively, GA may repress flowering in apple by upregulating expression of flowering repressor genes. A notable flowering repressor gene is *TFL1* (*TERMINAL FLOWER 1*). An apple *TFL1* gene, *MdTFL1-2*, was found responsive to both the presence of fruits and exogenous GA (Haberman et al. 2016), making it a potential candidate for GA-mediated repression of flowering. In Arabidopsis TFL1 competes with FT for binding to FD, and the binding of TFL1 and FD represses expression of downstream integrator flowering genes such as *LFY* and *AP1* (Ahn et al. 2006). Apple contains two genes related to the flowering repressor gene *TFL1*, *MdTFL1-1* and *MdTFL1-2*, and silencing of these genes in apple led to early flowering (Kotoda and Wada 2005; Szankowski et al. 2009).

Ultimately, the repression of flowering by GA in apple is expected to be driven by changes in the expression of those apple genes that are expected to have key roles in flowering.

GA and Alternate Bearing

Alternate bearing, also known as biennial bearing, is a widely spread production challenge in many perennial woody trees, including apple, pear, citrus, mango, apricot, pecan and avocado (Monselise and Goldschmidt 1982; Jonkers 1979; Mészáros et al. 2015). It is a phenomenon where fruit trees have “on” (heavy cropping) and “off” (light cropping) years alternatively. In the “on” year an excessive number of fruits are produced, even though the size and quality of the fruits are usually compromised. One of the major consequences is that floral induction is repressed by the presence of the heavy crop, resulting in no or few flowers/fruits the following year (“off” year). Alternate bearing is most extensively studied in apple. Among the most consumed and profitable apple cultivars in the world, ‘Honeycrisp’ and ‘Fuji’ are highly susceptible, and ‘Golden Delicious’ shows strong tendency to alternate bearing.

In apple, fruit development coincides with floral induction in the shoot apex. Based on the source/sink relationship between developing fruits and the adjacent shoot apex, two theories have been developed to explain the occurrence of alternate bearing. The first theory involves the competition for carbohydrates. Both developing fruits and the adjacent shoot apex are sinks for carbohydrates. It is proposed that developing fruits have a stronger sink strength than the shoot apex, and thus more carbohydrates are diverted into fruits, leading to a vegetative fate determination in the shoot apex (Wardlaw 1990). This hypothesis is supported by leaf removal experiments, where floral induction can be repressed by removal of the adjacent bourse shoot leaves, a condition that reduces

photosynthate availability (Fulford 1966; Elsysy and Hirst 2019). However, there is still no solid evidence supporting this model.

The second theory involves plant hormone GA. It has been shown that fruit diffusates from an alternately bearing apple cultivar have more GA activity than those from a regularly bearing apple cultivar (Hoad 1977). Isotope labeling experiments showed that a considerable amount of bioactive GAs is produced in immature seeds of developing fruits and can be subsequently transported into the adjacent bourse shoot apices, where shoots and flowers are formed for the following year (Luckwill et al. 1969; Ramírez et al. 2001). It is hypothesized that the seed-derived GAs are responsible for the repression of floral induction. This hypothesis is supported by previous studies in the seedless apple cultivar, 'Spencer Seedless'. Under normal conditions, 'Spencer Seedless' trees tend to bear annually; however, when seeds are produced through hand pollination, return bloom is reduced the following year (Chan and Cain 1967; Dennis and Neilsen 1999). Later fruit thinning experiments indicate that the existence of fruits did negatively impact flower formation in the bourse shoot apex (Gottschalk et al. 2021). Based on these findings, it is hypothesized that excessive GAs derived in seeds in "on" year represses flower formation, which results in few or no fruit formation the following year, thus an "off" year, but the direct role of seed-derived GAs on flowering is in question. A recent quantitative trait loci (QTL)-association study on an F1 population from a strong alternate bear ('Starkrimson') x regular bearer ('Granny Smith') cross provided some new evidence (Guitton et al. 2012). It is found that several GA-associated genes including four *MdGA2ox*, two *MdGA3ox* and three *MdGA20ox* genes and five DELLA-encoding genes, co-located with the QTLs responsible for alternate bearing, further supporting GA's role in alternate bearing.

GA in Abiotic Stress Response

In addition to its roles in plant growth and developmental processes, the GA signaling pathway is believed to be one of the survival strategies that plants employ under adverse conditions, as immediate enhancement or reduction in GA activity and signaling is necessary for plants to halt vegetative growth and better withstand or escape stresses (Colebrook et al. 2014). Early evidence for the GA-associated abiotic stress response was from the applications of plant growth retardants (Halevy and Kessler 1963; Gilley and Fletcher 1998; Vettakkorumakankav et al. 1999), the mode of action of which is primarily reducing internal GA levels by inhibiting GA biosynthesis (Rademacher 2000). Applications of these chemicals resulted in reduced growth but enhanced tolerance to drought and heat stresses. In contrast, a spray of GA to a dwarf barley line reversed its dwarf phenotype as well as inherent stress tolerance (Vettakkorumakankav et al. 1999). Later studies in Arabidopsis plants found that salinity and cold stresses trigger reduction of bioactive GA levels presumably through upregulation of *GA2ox* (Achard et al. 2006; Achard et al. 2008). Interestingly, a recent study found that touch, a mechanical stress, slowed down primary growth and delayed flowering time by reducing GA levels possibly through regulation of *AtGA2ox7*: touch induced expression of *AtGA2ox7*, and *ga2ox7* mutants was hyposensitive to this specific stress (Lange and Lange 2015). In addition, reduction of GA levels through *SlGA2ox7* is required for normal stomatal closure in response to drought in tomato (Shohat et al. 2021). GA is also involved in the escape from submergence and shade stresses likely through a different mechanism where GA levels are enhanced through the upregulation of GA biosynthetic genes *GA20ox* and/or *GA3ox* (Bailey-Serres and Voesenek 2010; Stamm and Kumar 2010).

The *GA2-oxidase* Gene Family

GA2ox enzymes comprise three distinct classes (Lee and Zeevaart 2005; Serrani et al. 2007). Class I and II GA2oxs specifically deactivate C19-GAs and are thus capable of inactivating bioactive GAs. In Arabidopsis, Class I enzymes include GA2ox1, GA2ox2, and GA2ox3, whereas Class II enzymes include GA2ox4 and GA2ox6 (*GA2ox5* is a pseudogene) (Table 1). Distinction between Class I and Class II enzymes is based on amino acid sequence and catalytic activity. Class I members (except GA2ox1) have multi-catalytic activity and can produce GA catabolites by further oxidation, whereas Class II members have mono-catalytic activity and so are not capable of producing GA catabolites (Thomas et al. 1999; Serrani et al. 2007). Class III comprises two additional GA2ox proteins, GA2ox7 and GA2ox8, which were shown to use C20-GAs (GA_{12} and GA_{53}), but not C19-GAs, as substrates (Schomburg et al. 2003) (Table 1). Based on this, they are likely involved in regulating early intermediates but not the degradation of the active GAs. Recently, two more Class III GA2ox proteins, GA2ox9 and GA2ox10, were characterized (Lange et al. 2020). Interestingly, it is shown that GA2ox9 can use C19-GAs as substrates.

GA2ox enzymes possess two conserved domains: the Non-haem dioxygenase N-terminal domain (Pfam: DIOX_N), which is a conserved domain of 2-oxoglutarate (2OG)/Fe(II)-dependent dioxygenases, and the domain with the 2OG and Fe(II)-dependent oxygenase activity (Pfam: 2OG-FeII_Oxy). The function of the N-terminal domain is not clear, but it was recently found that it has a role in forming the higher-order protein structures and interacting with its substrate GA (Takehara et al. 2020). The 2OG-FeII_Oxy domain contains active sites that interact with the substrates including 2OG, Fe(II) and GA (Huang et al. 2015; Takehara et al. 2020).

GA2ox genes are widely expressed throughout the plant life cycle, with *AtGA2ox2* and *AtGA2ox6* being the most highly expressed in Arabidopsis (Rieu et al. 2008; Li et al. 2019). In addition, *AtGA2ox6* is predominantly expressed in young seedlings and developing roots; *AtGA2ox2* is dominant in flower organs and siliques; *AtGA2ox1* transcripts are abundant in flowers; *AtGA2ox3* is found to be highly expressed in pollen; and *AtGA2ox4* was preferably expressed in developing seed and shoot apex. Analysis of loss-of-function mutants suggests that Class I and Class II *GA2ox* genes are functionally redundant: single knockout of each gene failed to confer apparent phenotypes, whereas GA-associated phenotypes were observed in the *atga2ox1/2/3/4/6* quintuple mutant, including early flowering, elongated hypocotyl, and seed abortion (Rieu et al. 2008). Additional mutant analyses revealed that *AtGA2ox4* may contribute to flowering and At*GA2ox2* to pistil elongation. The most prominent phenotype of the plants overexpressing *AtGA2ox7* or *AtGA2ox8* were dwarfism, indicating both genes are involved stem elongation, however, loss-of-function mutant analyses revealed that two genes do not have overlapping roles in seed dormancy (Schomburg et al. 2003). *AtGA2ox9* transcripts are abundant throughout the Arabidopsis life cycle, with the highest expression observed in 10-d root and shoot, while *AtGA2ox10* is predominantly expressed in siliques, with extremely low expression in other structures examined (Lange et al. 2020).

Table 1. GA2ox enzymes and their substrates

Class	GA2ox members	Substrates
I	GA2ox1, GA2ox2, and GA2ox3	C19-GAs (GA_1, GA_4, GA_9, GA_{20}, GA_{51}, GA_{34}, GA_{29}, GA_8)
II	GA2ox4 and GA2ox6	C19-GAs (GA_1, GA_4, GA_9, GA_{20})
III	GA2ox7, GA2ox8, GA2ox9 and GA2ox10	mainly C20-GAs (GA_{12} and GA_{53})

Regulation of *GA2ox*

Expression of *GA2ox* is tightly regulated predominantly at the transcriptional level to fine tune GA concentrations in response to both internal and external cues, and in some developmental contexts, upstream regulators have been identified. For example, the MADS-domain factor AGL15 (AGAMOUS-LIKE 15) directly upregulates *AtGA2ox6* in the developing embryo, through binding to the *AtGA2ox6* regulatory region (Wang et al. 2002, 2004). It was presumed that this results in the depletion of bioactive GAs and induction of dormancy. During seed germination in Arabidopsis, the bHLH factor PIL5 (phytochrome interacting factor 3-like 5) promotes *AtGA2ox2* expression, and its degradation in response to light allows GA to accumulate (Oh et al. 2006). In Arabidopsis and pea, seedling photomorphogenesis involves the promotion of *GA2ox* gene expression, and consequent GA depletion, by light (Alabadí et al. 2004; Reid et al. 2002; Zhao et al. 2007). In potato, tuber formation in response to short days is mediated by

increased *StGA2ox1* expression in the stolon meristem (Kloosterman et al. 2007). In Arabidopsis, short-term cold exposure results in upregulation of three *GA2ox* genes (*GA2ox1*, *GA2ox3* and *GA2ox6*) (Achard et al. 2008). This may involve transcriptional activation by the transcription factor CBF1 (C-repeat/DRE binding factor 1), because ectopic expression of *CBF1* in transgenic plants is sufficient to upregulate at least *GA2ox3* and *GA2ox6* (Achard et al. 2008). During seed dormancy in Arabidopsis, *GA2ox6* expression was strongly upregulated in response to cold, and this was dependent on *DOG1* (*DELAY OF GERMINATION 1*), which is one of the prominent genes that control seed dormancy (Kendall et al. 2011). Also in Arabidopsis, *GA2ox* genes are salt-responsive and for at least one, *AtGA2ox7*, induction is dependent on transcription factor DDF1 (DWARF AND DELAYED FLOWERING 1), which binds to a DRE (Drought-Responsive Element) on the *AtGA2ox7* promoter (Magome et al. 2008). In all these cases, it is presumed that the rapid deployment of *GA2ox* reduces GAs to halt plant growth, facilitating survival under stress conditions.

To maintain normal growth and development and to defend themselves against biotic and abiotic stresses, plants have developed mechanisms to modulate transcriptional/post-transcriptional activities and hormone signaling via a sophisticated interaction network. The versatility of GA signaling to control growth and development in response to external signals involves crosstalk with other hormones. Two mechanisms underlying this crosstalk have been proposed. In the first, DELLA proteins directly integrate multiple pathways through interaction with various transcription factors (Achard et al. 2006; Achard et al. 2008; Hou et al. 2010; Bai et al. 2012; Gallego-Bartolomé et al. 2012; Colebrook et al. 2014; Lor and Olszewski 2015). In the other proposed mechanism,

signals from various pathways regulate GA metabolic genes and bioactive GA levels through transcriptional regulation that does not involve DELLA proteins (van Huizen et al. 1995; Ross et al. 2000; Ozga et al. 2003). Whereas DELLA-dependent pathways have been well-characterized, how GA metabolic genes are potentially regulated by other pathways remains to be studied. Accumulating evidence shows that *GA2ox* genes become very important in this crosstalk. For example, in the Arabidopsis shoot meristem, a central domain of cytokinin activity promotes rapid cellular proliferation required for meristem maintenance and production of primordia anlagen (Hudson 2005). This is mediated through the promotion of the expression of *IPT* (*Isopentenyl-transferase*), a key cytokinin biosynthetic gene, by a KNOX (KNOTTED-LIKE HOMEOBOX) transcription factor SHOOT MERISTEMLESS (STM) (Yanai et al. 2005). GAs are synthesized in the new organ to promote the cell expansion required for the organ to assume its size and shape. Cytokinin promotes expression of *AtGA2ox2* and *AtGA2ox4* in a restricted domain at the boundary between the meristem and developing leaves (Yanai et al. 2005; Jasinski et al. 2005). This is expected to prevent diffusion of bioactive GAs into the meristem, where their effects might disrupt meristem structure (Jasinski et al. 2005). In Arabidopsis seedlings, auxin regulates hypocotyl elongation, and this effect is mediated at least in part through control of GA concentration via upregulation of five *AtGA2ox* genes (Frigerio et al. 2006). In *Sorghum bicolor*, ABA (abscisic acid) promotes seed dormancy via promoting expression of the presumed transcription factors *SbABA-INSENSITIVE 4* (*ABI4*) and *SbABI5*. These bind to the promoter of *SbGA2ox3*, a homolog of Arabidopsis Class I *GA2ox* genes, to increase its expression (Cantoro et al. 2013). In addition, ABA also interacts with GA to control hypocotyl elongation through a transcription factor

BBX21 (B-box zinc finger protein 21), an integrator of ABA signaling and light, targeting the promoter region of *AtGA2ox1* (Xu et al. 2017). The identified upstream TFs of *GA2ox* genes in plant development and growth, response to abiotic stress, and hormone crosstalk has been summarized in Table 2.

As discussed above, regulation of *GA2ox* occurs mainly at the transcriptional level where expression of this gene family is influenced by internal and external signals through transcription factors either directly or indirectly. Recently, it is reported that enzyme activity of a rice GA2ox can be enhanced through tetramerization in response to GA (Takehara et al. 2020), suggesting presence of regulation at the post-translational level.

Table 2. Upstream direct regulators of *GA2ox* genes

Transcription Factor	GA2ox promoter	Developmental Events	Reference
AtBBX21	*AtGA2ox1*	hypocotyl elongation	Xu et al., 2017
SbABI4, SbABI5	*SbGA2ox3*	seed dormancy	Cantoro et al. 2013
AtABI4	*AtGA2ox7*	GA-ABA antagonism	Shu et al. 2016
AtAGL15	*AtGA2ox6*	Embryo development	Wang et al. 2002, 2004
OsBZR1	*OsGA2ox3*	BR-GA interaction/cell elongation	Tong et al. 2014
AtDDF1	*AtGA2ox7*	salt response	Magome et al. 2008
ARBORKNOX1	Poplar *GA2ox4* and *GA2ox6*		Liu et al. 2015
ZmKNOTTED1	*ZmGA2ox1*	meristem maintenance	Bolduc and Hake 2009

Rationale and Justification

Many elements of GA metabolism and signaling have been strongly conserved during evolution of higher plants such as *Arabidopsis thaliana* and apple (*Malus x domestica* Borkh.). However, the effect of GA on floral induction is strikingly different. In apple, GAs presumably affect expression of flowering regulatory genes, but the identity of these potential genes and mechanism(s) of regulation is unknown. The repressive effect of GAs on flowering in perennial plants is not only an intriguing biological phenomenon, but also has great commercial importance. Our lack of knowledge has limited the development of more efficient methods to control flowering in apple, as well as the breeding of new cultivars with more desirable flowering traits. Apple trees typically have a long juvenility period (time between seed germination and first flowering) ranging from 5-12 years (Fischer 1994). Even though grafting young seedlings onto dwarf or semi-dwarf rootstocks shortens the juvenility stage, it still takes years for apple trees to gain the capacity of flowering, hindering genetic modification-based molecular and genetic studies on GA's role in flowering in apple. To circumvent this limitation, we took advantage of RNA-seq technology and regulation of GA catabolic genes *GA2ox* to probe into GA biology in apple and the annual model plant *Arabidopsis thaliana* with a specific focus on GA's role in flowering.

In this study, I addressed this lack of knowledge through the following three studies:

1) *Genetic mechanisms in the repression of flowering by gibberellins in apple (Malus × domestica Borkh.)*. Currently, there is little information available on gene expression in the apple shoot apex related to flowering, or on the genetic response to GAs. In this study, we carried out transcriptional profiling of apple shoot apices, during

the seasonal period in which floral initiation occurs. In addition, we examined how gene regulation is influenced by exogenous GAs. The analysis of GA-response genes identified components potentially influencing flowering, and we proposed a genetic mechanism of GA's repressive role in apple flowering.

2) Expression of GA2-oxidase homoeologs indicates both function divergence and redundancy in apple (Malus x domestica Borkh.). Taking advantage of this tight link between GA2ox and GA activity, we aimed to develop regulation of *GA2ox* as a model to evaluate GA activity and function in apple. The genomic organization of *GA2ox* genes were documented through analysis of two recently released high-quality apple genomes and our transcriptional datasets (above). We analyzed the developmental expression of *GA2ox* genes in apple trees and seedlings under normal conditions as well as GA-limiting conditions.

3) Developing a GA2ox toolkit in Arabidopsis thaliana. The Arabidopsis *GA2ox* has been relatively well studied, and we assessed the use of regulation of *GA2ox* as a model to understand GA function in this model plant. A prerequisite step was to develop *GA2ox* expression reporter lines and single knockout lines. By analyzing the phenotypes of the newly developed lines, we identified new functions of *AtGA2ox2* in plant growth and development.

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CHAPTER TWO

Genetic Mechanisms in the Repression of Flowering by Gibberellins in Apple (*Malus* × *Domestica* Borkh.)

Genetic Mechanisms in the Repression of Flowering by Gibberellins in Apple (*Malus* × *Domestica* Borkh.)

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ABSTRACT

Gibberellins (GAs) can have profound effects on growth and development in higher plants. In contrast to their flowering-promotive role in many well-studied plants, GAs can repress flowering in woody perennial plants such as apple (*Malus* x *domestica* Borkh.). Although this effect of GA on flowering is intriguing and has commercial importance, the genetic mechanisms linking GA perception with flowering have not been well described.

Application of a mixture of bioactive GAs repressed flower formation without significant effect on node number or shoot elongation. Using Illumina-based transcriptional sequence data and a newly available, high-quality apple genome sequence, we generated transcript models for genes expressed in the shoot apex and estimated their transcriptional response to GA. GA treatment resulted in downregulation of a diversity of genes participating in GA biosynthesis, and strong upregulation of the GA catabolic *GA2 OXIDASE* genes, consistent with GA feedback and feedforward regulation, respectively. We also observed strong downregulation of numerous genes encoding potential GA transporters and receptors. Additional GA-responsive genes included potential components of cytokinin (CK), abscisic acid (ABA), brassinosteroid, and auxin signaling pathways. Finally, we observed rapid and strong upregulation of both of two copies of a gene previously observed to inhibit flowering in apple, *MdTFL1* (*TERMINAL FLOWER 1*).

The rapid and robust upregulation of genes associated with GA catabolism in response to exogenous GA, combined with the decreased expression of GA biosynthetic genes, highlights GA feedforward and feedback regulation in the apple shoot apex. The finding that genes with potential roles in GA metabolism, transport and signaling are

responsive to GA suggests GA homeostasis may be mediated at multiple levels in these tissues. The observation that *TFL1*-like genes are induced quickly in response to GA suggests they may be directly targeted by GA-responsive transcription factors and offers a potential explanation for the flowering-inhibitory effects of GA in apple. These results provide a context for investigating factors that may transduce the GA signal in apple and contribute to a preliminary genetic framework for the repression of flowering by GAs in a woody perennial plant.

APPENDIX

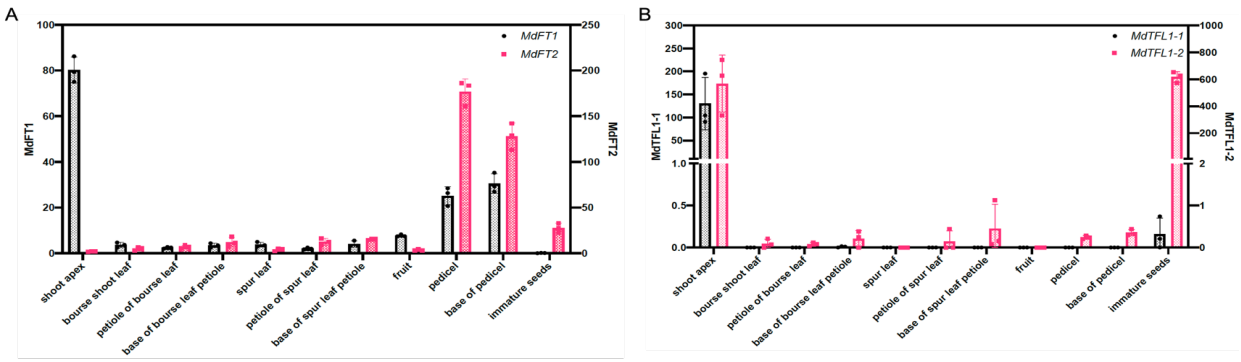

Figure 5. Expression of two pairs of floral genes, *MdFT1*/-2 and *MdTFL1*/-2, in 'Gala' spur structures. A. Relative expression of *MdFT1* (black, plotted on the left y axis) and *MdFT2* (pink, plotted on the right y axis); B. Relative expression of *MdTFL1-1* (black, plotted on the left y axis) and *MdTFL1-2* (pink, plotted on the right y axis). Samples were collected at 40 days after full bloom. Dots on column bars represent individual values from three biological replicates, and error bars represent standard deviation among three replicates.

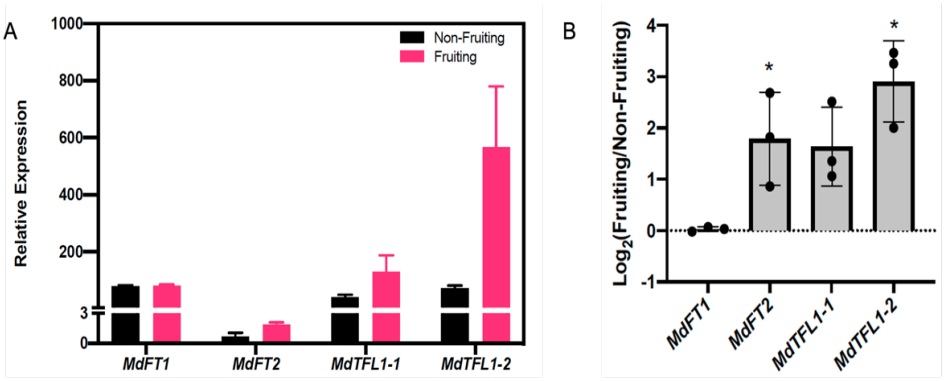

Figure 6. Relative expression of *MdFT* and *MdTFL1* in the 'Gala' shoot apex in response to the presence of fruits. A. Relative expression of genes in the bourse shoot apex of spurs with (pink) or without (black) fruits. B. $\log_2$ (fold change of ratio between expression in fruiting and non-fruiting samples). Dots on column bars represent individual values from three biological replicates. Error bars represent standard deviation among three replicates. The asterisk symbols denote that difference between expression in fruiting and that in non-fruiting samples was significant ($p < 0.05$).

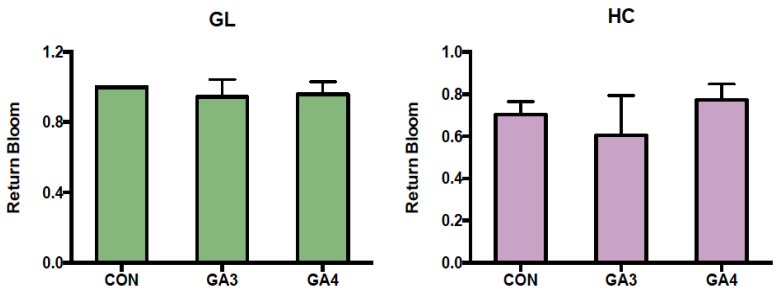

Figure 7. Return bloom affected by GA_{4+7} (shown as GA_4) and GA_3 sprays in 'Gala' (GL, green bars) and 'Honeycrisp' (HC, pink bars). Y axis represents percentage of blooming spur shoots (1 = 100%). Error bars represent standard deviation among three replicates.

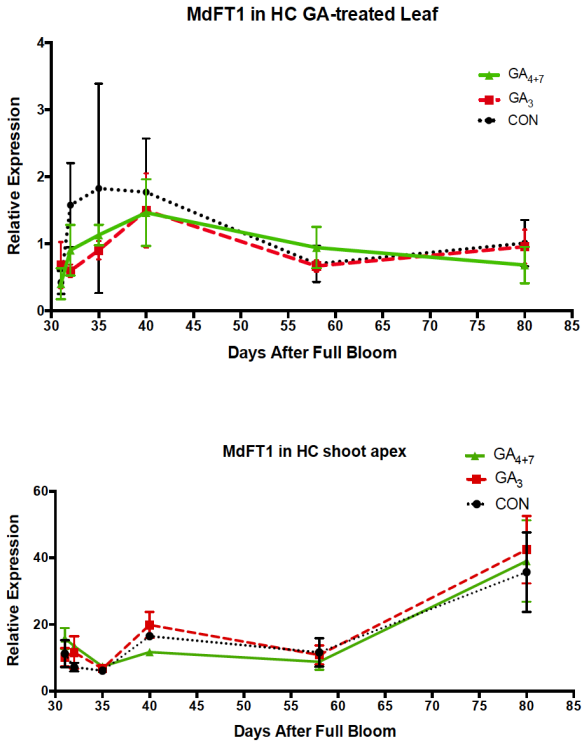

Figure 8. Expression of *MdFT1* in the 'Honeycrisp' bourse shoot leaf and shoot apex in response to GA_{4+7} (green) and GA_3 (red). CON, control.

CHAPTER THREE

Expression of *GA2-oxidase* Homoeologs Indicates both Function Divergence and Redundancy in Apple (*Malus x domestica* Borkh.)

ABSTRACT

In higher plants, the major mechanism for elimination of bioactive gibberellins (GAs) and resetting of GA signaling is through catabolism by a family of GA2-oxidases (GA2ox). Expression of *GA2ox* genes is tightly regulated by internal and external cues. In this study, using two newly available high-quality apple reference genomes as well as transcriptome datasets derived from the shoot apex, we carried out a genome-wide census and identified a total of 16 *GA2ox* genes in the apple genome, representing eight pairs of homoeologous genes. They are named uniquely (from *MdGA2ox1A*/*-1B* to *MdGA2ox8A*/*-8B*) to reflect their genomic organization and phylogenetic relationship. Using highly specific Taqman gene assays for 14 loci, we found that two genes (*MdGA2ox1B* and *MdGA2ox6B*) were ubiquitously expressed in apple, suggesting their potential roles in a wide spectrum of growth and development processes. We observed structure-specific expression for most genes. We observed both distinct and similar spatial and developmental expression patterns between individual members of homoeologous pairs under various conditions, indicating gene divergence and potential function redundancy. Interestingly, a pair of homoeologous genes (*MdGA2ox2A* and *MdGA2ox2B*) were predominantly expressed in the shoot apex, and they were readily induced in this specific structure by both exogenous GA and presence of the source of endogenous GA during the period of floral induction, indicating their potential involvement in GA-mediated repression of flowering. Altogether, these findings provide biological and evolutionary insights into GA biology in apple, particularly in floral induction.

Introduction

Gibberellins (GAs) are a class of plant hormones found in all vascular plants. To date, a total of 136 GA forms have been identified, among which GA_1, GA_3, GA_4 and GA_7 appear to be the bioactive forms (reviewed in Hedden 2020). The early steps of GA biosynthesis involve the production of *ent*-kaurene from trans-geranylgeranyl diphosphate (GGPP) by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) and the production of the initial GA form GA_{12} by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). The later steps involve sequential oxidation of non-bioactive C20-GA precursors into active C19-GAs by two groups of 2-oxoglutarate-dependent dioxygenases (2-ODDs), GA20-oxidase and GA3-oxidase. In gymnosperms and angiosperms, both active and inactive GAs can be catabolized by a distinct group of 2-ODDs, the GA2-oxidase (GA2ox). Even though bioactive GAs can be deactivated through other pathways (Schneider and Schliemann 1994; Zhu et al. 2006; Varbanova et al. 2007), GA2ox catabolism is the predominant route for elimination of bioactive GAs and resetting of GA signaling (Rieu et al. 2008). In plant tissues, GA levels can be maintained through a homeostatic mechanism involving transcriptional regulation of the biosynthetic genes *GA20ox* and *GA3ox* and the GA catabolic genes *GA2ox*. This regulation is mostly at the transcriptional level, although a recent different mechanism of GA homeostasis involving post-translational regulation of GA2ox has been found in rice (Takehara et al. 2020).

GA2ox enzymes (gibberellin 2-β-dioxygenases; EC 1.14.11.13) comprise three distinct classes (Lee and Zeevaart 2005; Serrani et al. 2007). Class I and II GA2ox specifically target C19-GAs and are capable of inactivating bioactive GAs by

hydroxylation at C-2 (Rieu et al. 2008). Class III enzymes mainly target the early intermediate C20-GAs, GA_{12} and GA_{53}, as substrates but not the bioactive GAs (Schomburg et al. 2003). Distinction between Class I and Class II enzymes has been based on amino acid sequence and catalytic activity (Thomas and Hedden 1999; Serrani et al. 2007; Ubeda-Tomás et al. 2006). It is estimated that C19-GA2ox have evolved prior to the establishment of gymnosperms followed by the appearance of C20-GA2ox (Class III) and subclassification of C19-GA2ox (into Classes I and II) before angiosperms (Yoshida et al. 2020; Huang et al. 2015; Hernández-García et al. 2021). Gene copy number within each class has expanded in further duplication events in angiosperms (Yoshida et al. 2020). In Arabidopsis where GA catabolism has been extensively studied, Class I enzymes include GA2ox1, GA2ox2, and GA2ox3, whereas Class II enzymes include GA2ox4 and GA2ox6 (*GA2ox5* is a pseudogene). Class III comprises GA2ox7, GA2ox8 and the newly characterized GA2ox9 and GA2ox10 (Lange et al. 2020).

In apple (*Malus x domestica* Borkh.), one of the most widely grown and economically important temperate tree fruits, GA influences numerous aspects of development and physiology that condition production traits including yield and fruit quality. A notable example is GA's role in floral induction. In the annual herbaceous plant *Arabidopsis thaliana* (Arabidopsis), GAs promote floral induction by activating the expression of genes such as *LFY* (*LEAFY*) and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CO 1*) that direct flower formation (Blázquez et al. 1998; Moon et al. 2003). However, in apple, as well as many other tree fruit species, exogenous GAs generally have a negative effect on floral induction (Tromp 1982; Bukovac et al. 1986; Southwick et al. 1995; Bertelsen and Tustin 2002; Goldberg-Moeller et al. 2013; Zhang

et al. 2019). In addition, in apple, seed-derived GAs are thought to repress floral initiation on adjacent bourse shoots, contributing to one of the major production challenges, alternate bearing. In apple, floral induction coincides with fruit development. When many fruits are present in a tree (i.g. ‘on’ year), excessive GAs are produced in the seeds of developing fruits and subsequently transported into the adjacent bourse shoot apex (Luckwill et al. 1969). It is thought that GA represses floral induction in the shoot apex, resulting in few or no fruits the following year (i.g. ‘off’ year), which could cause significant economic loss to apple growers and industry without proper crop management. Despite its importance, little is known about GA activity and GA signaling pathway in apple.

Quantitative trait loci (QTL)-association analysis of an F1 population from a strong alternate bear (‘Starkrimson’) x regular bearer (‘Granny Smith’) cross revealed that GA is involved in alternate bearing through regulation of GA-associated genes (Guitton et al. 2012). It is found that four *MdGA2ox* genes (two of which are now named *MdGA2ox1B* and *MdGA2ox2B*) along with several other genes co-located with the QTLs responsible for alternate bearing, supporting GA's role in alternate bearing. Previously, we identified four *MdGA2ox* genes, now named as *GA2ox1A*, *-1B*, *-2A* and *-2B*, that were rapidly and persistently induced by exogenous GA_{4+7} as well as by the presence of fruits - the major source of bioactive GA in the apple shoot apex during the anticipated period of floral induction (Zhang et al. 2019; Gottschalk et al. 2021). The results prompted us to develop *MdGA2ox* as a model to study GA’s roles in apple growth and development. We propose that these genes are responsible for regulating GA activity in the shoot apex during floral induction.

Taking advantage of two newly available high-quality apple reference genomes (Daccord et al. 2017; Sun et al. 2020) and our transcriptome datasets derived from the 'Gala' shoot apices (Zhang et al. 2019), we identified the canonical *GA2ox*-like genes in the apple genome, examined their genomic organization, and assessed gene divergence and function redundancy by comparing their spatial and developmental expression profiles in various structures under several conditions. With this study, we hope to provide a biological and evolutionary perspective into GA biology in apple with an emphasis on floral induction.

Results

Census and genomic organization of apple *GA2ox* genes

As a first step to comprehensively identify canonical *GA2ox* genes in apple, we carried out a census of *GA2ox* genes as annotated in the genome of a doubled-haploid derivative of cv. 'Golden Delicious' ('GDDH13'; Daccord et al. 2017). We used the BLAST sequence homology search tool (Altschul et al. 1990) with 14 protein sequences from Arabidopsis, rice, pea or bean cataloged as "Gibberellin 2-beta-dioxygenase" in the ExPASy Enzyme database as queries (S1 file). All apple sequences showing significant homology (S2 file) were reciprocally used as queries to identify more distantly related sequences from Arabidopsis (S3 file). We additionally used a Hidden-Markov-Model-based approach (HMMer; Johnson et al. 2010) with sequence motif queries derived from the conserved domains of the 14 cataloged GA2ox proteins (S1-3 Tables). However, HMMer analysis did not result in identification of additional homologs. The most highly scoring protein sequences from both apple and Arabidopsis were subjected to phylogenetic analyses (Fig. 9A).

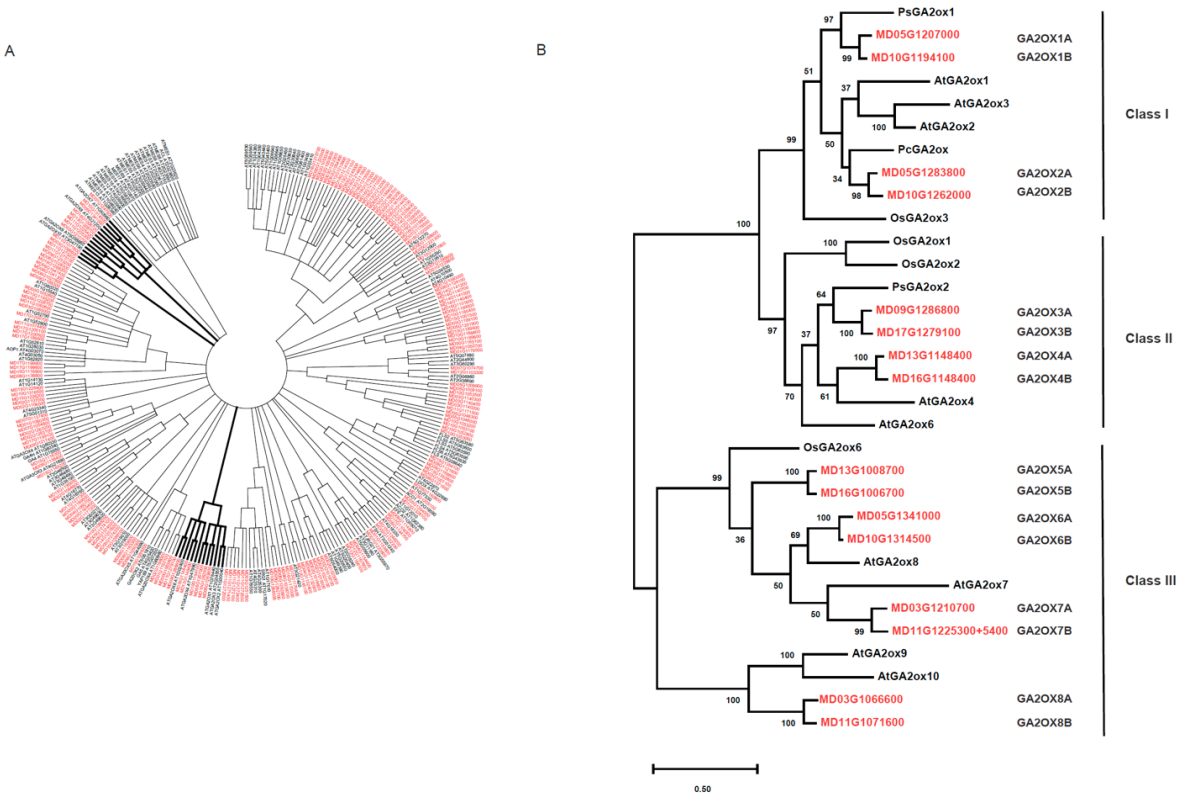

Figure 9. Phylogenetic analyses of GA2ox-related protein sequences. A) Sequences determined through BLAST. Apple sequences are shown in red; Arabidopsis or other plant sequences are shown in black. Clusters containing known Arabidopsis GA2ox proteins are highlighted in bold. B) Phylogenetic tree of apple GA2ox (highlighted in red) with their homologs in model plant species.

We found that the known Arabidopsis GA2ox sequences were represented by three clades, one containing AtGA2ox1-AtGA2ox4 and AtGA2ox6 along with eight apple sequences, one containing AtGA2ox7 and AtGA2ox8 and seven apple sequences, and one containing two newly characterized GA2oxs, AtGA2ox9 and AtGA2ox10 (Lange et al. 2020), along with two apple sequences. The AtGA2ox1/2/3/4/6 clade was further defined by two subclades, one containing AtGA2ox1-3 and four apple sequences, and the other containing AtGA2ox4 and AtGA2ox6 and four apple sequences. The 17 apple sequences included in these three clades were also those that showed the strongest homology with each of the individual GA2ox query sequences, as expected (Fig. 9A). The

next highest scoring apple sequences identified via blast were MD13G1170400, whose closest Arabidopsis homolog is LBO1 (LATERAL BRANCHING OXIDOREDUCTASE 1), and MD07G1299900 and MD01G1228800, both most closely related to Arabidopsis DMR6 (DOWNY MILDEW RESISTANCE 6) (Fig. 9A). These collective results suggested that the GDDH13 reference genome annotation included 17 canonical GA2ox sequences.

To support annotation of the apple genome and examine potential allelic diversity within these *MdGA2ox* genes, we analyzed the 17 gene models annotated for both reference genomes ('GDDH13' and 'Gala' phased diploid genome) with respect to transcript models derived from RNA-seq-based profiling of the shoot apex of 'Gala' (**S4 and 5 files**; Zhang et al. 2019). Two reference genomes were generally consistent with one another and with our results, with a few exceptions (Fig. 10). First, the 'Gala' genome supports a more extensive and distinct first exon of MD09G1286800 (designated as *MdGA2ox3A*; below). Second, the 'Gala' genome and our transcriptome data support that the transcribed region of MD16G1148400 (designated as *MdGA2ox4B*) is more extensive and contains a third exon. Third, the 'Gala' genome supports three exons in the transcribed region of MD05G1341000 (designated as *MdGA2ox6A*), rather than four as annotated in the GDDH13 genome. Fourth, the 'Gala' genome supports a more extensive transcribed region as well as a distinct third exon at the MD03G1210700 locus (designated as *MdGA2ox7A*). Finally, we identified a single transcript model including both MD11G1225300 and MD11G1225400 in the 'Gala' shoot apex. We verified that these two distinctly annotated loci generate a common transcript with an atypically long (~5.9 kb) intron using a Taqman PCR primer-probe set (Fig. 10 and S4 Table). The 'Gala' genome also indicates the presence of indels in the transcribed region of one or both

alleles at three loci (designated as *MdGA2ox3A*, *MdGA2ox7B* and *MdGA2ox8A*), however, these indels were not supported by our transcriptome data (not shown). In addition, additional transcript models were identified in the transcriptome datasets for several of the *MdGA2ox* genes. However, most of these isoforms contained regions that were introns in the major expressed isoforms, exhibited premature stop codons, and likely resulted from imprecise or incomplete splicing (Fig. 10 and S5 Table).

Canonical GA2ox proteins comprise two highly conserved peptide sequences: an amino-terminal segment found within proteins with 2-oxoglutarate/Fe(II)-dependent dioxygenase activity (DIOX_N) and a carboxyl-terminal segment that define members of the 2OG-Fe(II) oxygenase superfamily (2OG-FEII_Oxy). We found that 20 transcript models, representing 16 genes, could encode a protein containing both domains (Fig. 10 and S1-3 Tables). Two genes, MD05G1207000 and MD13G1008700, produced detectable levels of apparently unspliced transcripts in which premature translation termination would lead to loss of the 2OG-FEII_Oxy domain. The DIOX_N domain was absent in the transcript model for MD09G1286800 annotated in the GDDH13 genome, however, both domains were present according to the gene model annotated in the 'Gala' genome. The tandem reference genes MD11G1225300 and MD11G1225400 encode a DIOX_N domain and 2OG-FEII_Oxy domain, respectively, further supporting that these loci represent one gene (*MdGA2ox7B*). The 2OG-FEII_Oxy domain comprises the catalytic core that interacts with 2-oxoglutarate (including the conserved residues His, Asp, and His) and Fe^{2+} (Arg, Ser) (Huang et al. 2015). All the major gene models contain these conserved residues at the respective positions (**S5 Table**).

Based on this data, we concluded that there are a total of 16 canonical *GA2ox*-like genes in the apple genome. A refined phylogenetic tree of apple *GA2ox* genes including the 16 query *GA2ox* genes is shown in Figure 9B. This phylogeny suggested that these genes represent eight pairs of duplicated genes, consistent with their annotated chromosomal locations within the apple genome and syntenic relationship among apple chromosomes (Daccord et al. 2017). We named these apple *GA2ox* genes based on their phylogenetic relationship and genomic organization (Fig. 9B). Four (*MdGA2ox1A*, *-1B*, -2A, and *-2B*) are clustered in Class I, four (*MdGA2ox3A*, *-3B*, *-4A* and *-4B*) in Class II, and eight (*MdGA2ox5A*, *-5B*, *-6A*, *-6B*, *-7A*, *-7B*, *-8A* and *-8B*) in Class III (Fig. 9B).

GA feedforward regulation of apple *GA2ox* genes

In the present study, to further evaluate the expression of *MdGA2ox* genes and their response to GA in the apple shoot apex, we treated apple trees with a commercial formulation of GA_3 at 30 days after full bloom (DAFB), within the anticipated period of floral initiation. Expression of 14 genes was monitored by quantitative PCR, two days after application (Fig. 11). Most of the genes, including the four previously identified genes, were expressed to higher levels in the GA-treated plants. This upregulation by GA at this stage was most striking for C19-GA-specific *MdGA2ox1A*, *-1B*, *-2A*, *-2B*, *-4A* and *-4B* (Fig. 11).

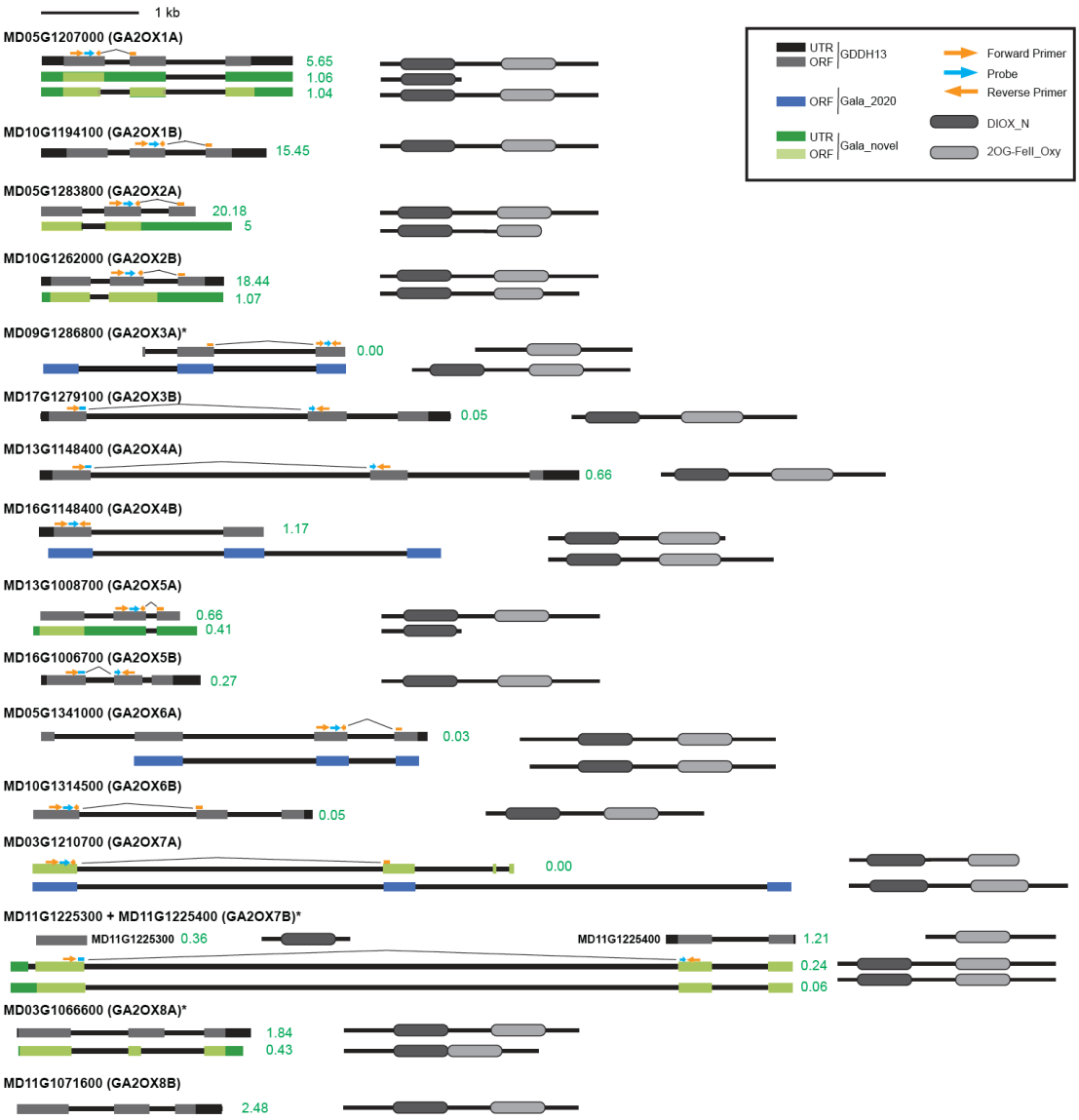

Figure 10. *MdGA2ox* gene, transcript, and protein models in the apple genome. Primary gene models annotated in the 'GDDH13' are shown in black/grey. The gene models annotated in the 'Gala' genome are mostly consistent with the 'GDDH13' genome, except for four loci (*MdGA2ox3A*, *-4B*, *-6A* and *-7A*) where 'Gala' models are given separately in blue. Additional transcript models from the 'Gala' shoot apex are shown in green. Untranslated regions (UTRs) (black or dark green) and exons (grey, light green or blue) are indicated as boxes. A scale bar for transcript models is given on the top. Positions of Taqman primers (orange) and probes (light blue) were shown in arrows and were not scaled proportionally. At least one of the three oligo sequences spanned one exon-intron junction (indicated by broken lines), except for *MdGA2ox4B*. Expression levels (FPKM, Fragments Per Kilobase of transcript per Million mapped reads) of each transcript in the 'Gala' shoot apex are shown in green next to the transcript models. *: loci where the 'Gala' genome supports the presence of an indel in one or both alleles that

Figure 10. (cont'd)
contradicts with our transcriptome data. Specifically, the 'Gala' genome supports a 63-bp deletion in one of the two alleles at the locus of *MdGA2ox3A*, and it is located within the binding region of the Taqman forward primer and probe (not shown). At the locus of *MdGA2ox7B*, this genome indicates there are two deletions (2 bp and 19 bp) within the probe binding region in both alleles, while at the locus of *MdGA2ox8A*, there is a long (~510 bp) insertion followed with short deletion in the first exon of both alleles. However, our transcriptome data did not support the presence of these indels. And, we would not have been able to detect the expression of *MdGA2ox7B* with the existent Taqman gene assay if the deletions were present in the materials used in this study.

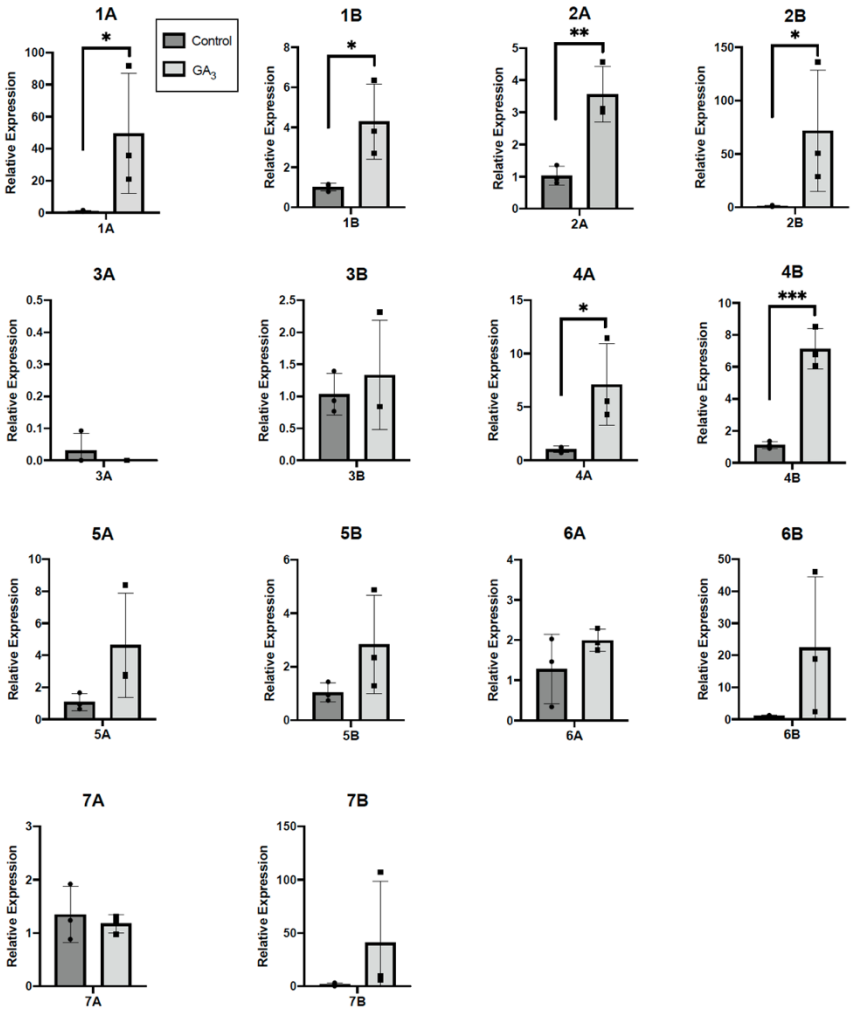

Figure 11. GA feedforward regulation of *MdGA2ox* genes in the bourse shoot apex. 'Gala' trees were either treated with 400 ppm of GA_3 or water (control) at 30 days after full bloom, and the bourse shoot apices were collected at two days after treatment. Black dots represent individual values from three biological replicates (the same below). An error bar indicates standard deviation among three biological replicates, while asterisks denote statistical significance (*, $p < 0.05$; **, $p < 0.01$) (the same below).

Developmental expression pattern of apple *GA2ox* genes

To evaluate developmental regulation of the *MdGA2ox* genes, we firstly monitored their expression within various structures that comprise the condensed shoots called spurs at 40 DAFB (Fig. 12). The spur structure comprises spur leaves, arising from primordia generated the previous season, the so-called bourse shoot generated in the present season, and may also include developing fruits, originating from flower buds (Fig. 12A). Among these structures, the bourse shoot apex is where the vegetative or mixed (both vegetative and floral) shoots for the next season are formed. As 'Gala' apple trees are typically spur-bearing and have an extremely high flowering rate on spurs (~100%; Zhang et al. 2019), the shoot apices collected were supposedly destined to develop into mixed shoots (both flowers and vegetative shoots).

In most of the structures, expression of at least half of the genes was easily detectable (Fig. 12B). The exception was the fruit flesh, where expression of only a small subset of the genes (*MdGA2ox3B*, *-5A*, *-5B*, *-6B* and *-7A*) was detected, and mostly at low levels. While *MdGA2ox1B* and *-6B* were expressed in all the spur structures examined, *MdGA2ox2B* and *MdGA2ox3A* were the most apparently weakly expressed genes in almost all structures. Interestingly, all the *MdGA2ox* genes showed strong structure-specific expression. The shoot apex of the fruiting spur showed a high expression of *MdGA2ox2A*/*-2B*. Expression of *MdGA2ox2B* seems to be confined in the shoot apex, and it was either undetectable or at a low level in other structures. Even though *MdGA2ox1A*/*-1B* were differentially expressed in the shoot apex in response to GA (Zhang et al. 2019 and Fig. 11), they were not preferably expressed in this particular structure without being treated. Instead, *MdGA2ox1A*/*-1B*, along with three other genes

(*MdGA2ox3B*, *-4A* and *-4B*), were predominantly expressed in immature seeds. A further dissection of immature seeds revealed that this high expression was all confined in the seed coat in the stage examined (33 DAFB) (Fig. 12C). Otherwise, *MdGA2ox1A* and *MdGA2ox1B* also had a relatively high expression in the base of spur leaf petiole. *MdGA2ox4B* was preferably expressed in fruit-associated structures (except for fruit flesh). Both *MdGA2ox6A* and *MdGA2ox6B* were preferably expressed in leaf and petiole. *MdGA2ox7A* and *MdGA2ox7B* had a relatively high expression in petiole-associated structures, and they were barely expressed in fruit-related structures. Expression of *MdGA2ox3A*, *-6A* and *-6B* was either not or barely detectable in the shoot apex.

To assess the potential effect of crop load on the expression of *MdGA2ox*, we also investigated the structures of the spurs without fruits (Fig. 12B). The expression patterns in non-fruiting spurs were mostly similar to that in fruiting spurs. However, the presence of developing fruits seemed to have an effect on most of the genes in at least one structure (Fig. 12B). It is mostly obvious in the shoot apex, where three out of the seven gene pairs, *MdGA2ox1A/-1B*, -2A/-2B and -5A/-5B, were significantly upregulated from 4 to 16 folds by the presence of fruits (S1 Fig.). It is worth noting that both crop load and exogenous GA upregulated the expression of *MdGA2ox1A*/*-1B* and *-2A*/*-2B* in the shoot apex (S1 Fig. and Fig. 11). However, *MdGA2ox4A*/*-4B*, which were significantly induced by exogenous GA, did not respond to the presence of crop load, and *MdGA2ox5A*/-5B, which were induced by crop load, were not significantly differential expressed in response to GA (S1 Fig. and Fig. 11).

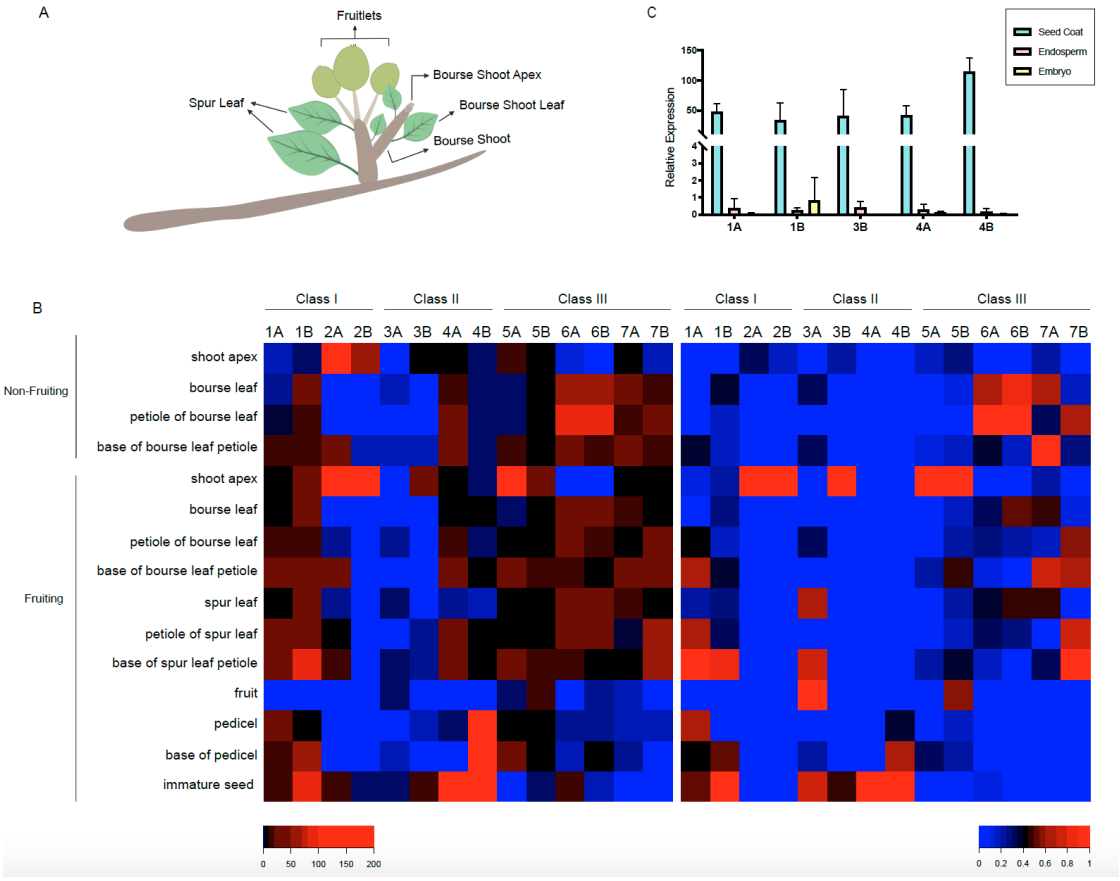

Figure 12. Expression profiles of *MdGA2ox* genes in spur structures. A. Illustration of an apple spur - the primary flowering unit of 'Gala' apple. B. Expression of *MdGA2ox* in the structures of spurs with (fruiting) and without fruits (non-fruiting). The heat map on the left represents expression values derived from quantitative PCR and relative to an apple *Actin* gene. To better show the tissue-specific expression, the heat map on the right represents the ratios of relative expression to the strongest expression for each gene among these structures. Color keys at the bottom indicate expression levels from high (red) to low (blue). C. High expression of a subset of genes in the seed coat. The x axis represents the five genes studied here, while the y axis represents their relative expression in seed coat (blue), endosperm (pink) and embryo (yellow).

We also evaluated the expression of *MdGA2ox* in various structures dissected from rapidly growing seedlings that were ~6 weeks old and had produced 9-11 true leaves (Fig. 13). These structures included the shoot apex (containing leaf primordia < 5 mm in length), and younger structures taken from the upper section of the seedling (leaf, petiole, base of the petiole, stem node and internode) as well as older structures from the central

section of the seedling (leaf, petiole, base of the petiole, stem node and internode). In addition, we analyzed the stipules pooled from the younger and older sections (Fig. 13).

Like the spur, strong expression of at least half of the *MdGA2ox* genes was detected in most of the structures (Fig. 13). The shoot apex saw high expression of *MdGA2ox2A* and -7B. Several genes, including *MdGA2ox1B*, *-5A*, *-5B*, *-6B* and *-7A*, were preferably expressed in the young leaf. Most genes had low or no expression in the corresponding young petiole. *MdGA2ox2A* was expressed to a high level in the base of young petioles. *MdGA2ox3A* and *-3B* were preferably expressed in the young internode and node. While some genes had similar expression patterns in young and old structures, some genes, however, preferred one over another. For example, the expression of *MdGA2ox1A* was only detected in older structures with the highest in the older petiole. The genes that were weakly expressed in spur structures, however, were expressed to moderate levels in several seedling structures. For example, *MdGA2ox3A*, whose expression was barely detected in spur structures, was predominantly expressed in younger internodes and nodes in seedlings. Consistently, *MdGA2ox1B* and *-6B* were expressed in all the seedling structures examined. In contrast, *MdGA2ox7A* and *-7B* were the most weakly expressed genes.

To summarize, the expression of *MdGA2ox* varies with developmental stages (in this case, adult tree structures and seedling structures). We found *MdGA2ox1B* and *-6B* are ubiquitously expressed in all the apple structures studied, suggesting their involvement in a variety of GA-mediated processes (Figs. 12 and 13).

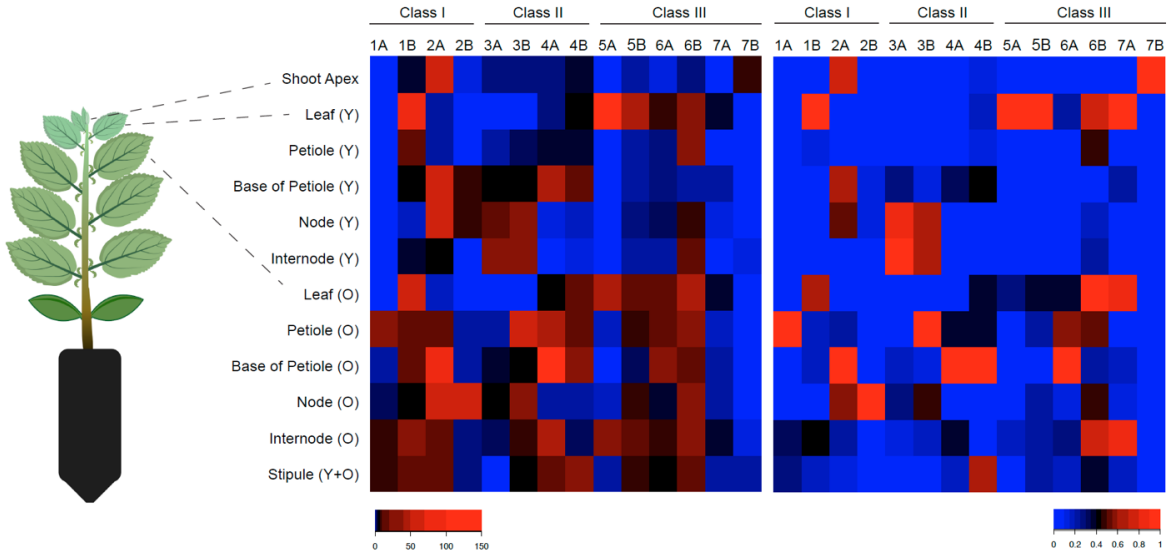

Figure 13. Expression profiles of *MdGA2ox* in apple seedling structures. O, old; Y, young. Old leaf was the first fully expanded leaf; young leaf was the first leaf immediately above the first fully expanded leaf. Old or young node was the one at the base of the corresponding old or young leaf. Old or young internode was the one immediately below the old or young leaf. Old or young petioles, base of petioles, and stipules were exercised from the corresponding old or young leaf. Old and young stipules were pooled together. Again, the heat map on the left represents relative expression, whereas the heat map on the right represents the ratios of relative expression to the strongest expression for each gene among these structures to better show the tissue-specific expression. Color keys at the bottom indicate expression levels from high (red) to low (blue).

Diurnal response and expression under stress conditions

Those whose expression patterns are highly correlated may have conserved or similar functions. It prompted us to examine gene divergence or function redundancy by looking more into their expression patterns but under GA-limiting conditions, including dark/light, abrupt water deficit and salinity. Some genes are subject to diurnal rhythms and are only expressed in specific structures during specific time periods within a day. Here we documented their expression dynamics in the seedling shoot apex and the first fully expanded leaf within 25 hours, spanning both the light and dark cycles (Fig. 14). The structure-specific expression of 14 *MdGA2ox* genes was consistent with the results in Figure 5. Specifically, *MdGA2ox2A*, *-2B*, *-3A*, *-3B*, and *-7B* had much higher expression

levels in the shoot apex than that in the first fully expanded leaf (corresponding to the old leaf in Fig. 13**)**, whereas *MdGA2ox1B*, *-5A*, *-6B*, and *-7A* were more highly expressed in the leaf. Despite the structure-specific expression, most genes exhibited various levels of diurnal response in both structures, except for *MdGA2ox2A*/*-2B* whose expression remained almost undetectable in the leaf and showed no response to the light and dark cycles (Fig. 14).

In general, there was no consistent diurnal pattern for all genes in either structure. However, when comparing the expression profiles of homoeologous gene pairs, we did notice that some gene pairs showed similar expression patterns, for example, *MdGA2ox5A/-5B* in both structures, *MdGA2ox1A*/*-1B* and *MdGA2ox2A*/*-2B* in the shoot apex, suggesting possibly overlapping function between pairs. Otherwise, the rest of the genes had distinct expression profiles, which strongly implies sub-functionalization or neofunctionalization of these homologous pairs in the process of evolution.

For most genes, expression at 1 h and 25 h was at similar levels in both structures examined in this study. The exceptions were *MdGA2ox3B* and *-7B* in the shoot apex. Both genes had a relatively high expression at 25 h in contrast with that at 1 h. It is worth knowing that the diurnal rhythm of plants generates endogenous oscillations with a period of about, but not exactly, 24 h. These genes may be involved in certain developmental process(es) which cause the difference in expression at the same time within two days.

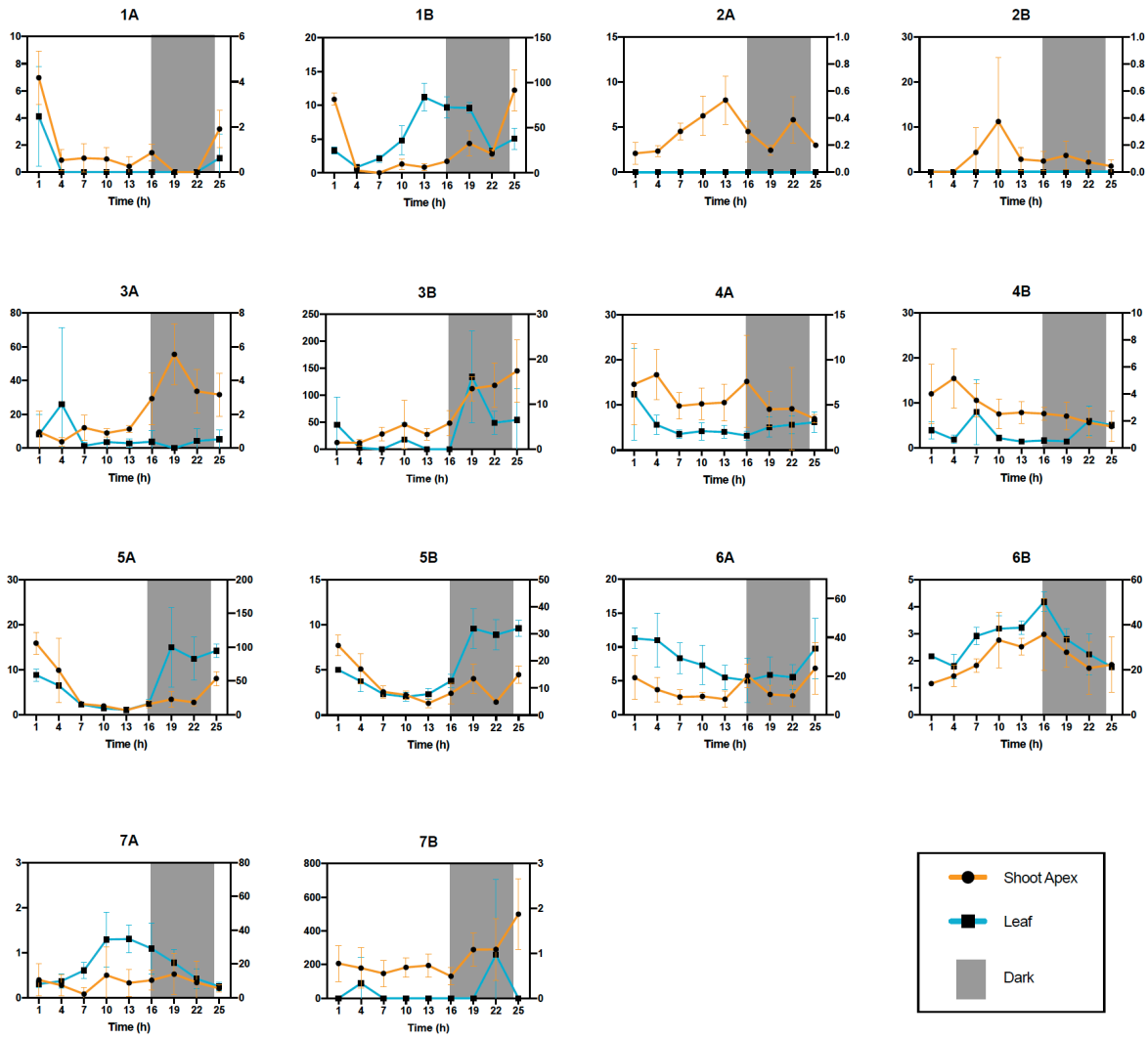

Figure 14. Diurnal expression of *MdGA2ox* genes in the shoot apex and first fully expanded leaf in seedlings. Shoot apices and the first fully expanded leaves were exercised from rapidly growing seedlings at nine time points within 25 hours, i.e. 8 am, 11 am, 2 pm, 5 pm, 8 pm, 11 pm, 2 am, 5 am and 8 am, corresponding to 1 h, 4 h, 7 h, 10 h, 13 h, 16 h, 19 h, 22 h and 25 h (x axis) after the lights were first turned on the experiment date. The y axis represents relative expression. Relative expression in the shoot apices and leaves is represented with orange and blue lines, respectively. The grey box in each graph indicates the period of dark.

We also evaluated their expression in the first fully expanded leaves of apple seedlings exposed to acute water loss (air dry). As water content dropped dramatically over time in the air-dry leaves (Fig. 15A), gene response was observed as early as at 2 h after treatment both in leaf blades and petioles (Figs. 15B and S2 Fig.). It was consistent

that the expression of most genes (except *MdGA2ox3A*, *-4A* and *-4B*) was upregulated by water deficit within 2 h after treatment in the leaf blades (Fig. 15B). While some of the genes were not responding in petioles at 2 h and either remained non-responding or were downregulated at 6 h, 2 hours after the water content reached its lowest point, *MdGA2ox1A*, *-3B* and *-6B* were consistently upregulated at 2 h and were not obviously responding at 6 h (S2 Fig.). These findings suggest that the apple *GA2ox* genes are fast responders to acute water loss, at least in apple leaves, and that a subset of them is turned on, likely to limit GA activities under this type of stress. The changes in expression of some genes in hydrated leaf blades and petioles did not always remain mild such as the high expression or upregulation of *MdGA2ox4A* at 2 h and 6 h in leaf blades, that of *MdGA2ox4A*, *-5A* and *-5B* at 6 h in petioles. This high expression cannot be fully explained by their diurnal response (Fig. 14), suggesting some unknown response may exist. The expression of two pairs of *MdGA2ox* (*MdGA2ox2A*/*-2B*, *-7A*/*-7B*) was not detected in leaf blades or petioles, even under water deficit stress, and thus not included in the figures. Again, we noticed that most homoeologous pairs, including *MdGA2ox1A*/*-1B*, *-4A*/*-4B*, *-5A*/*-5B* and *-6A*/*-6B*, shared similar expression profiles in both structures under water deficit stress, while *MdGA2ox3A* and *-3B* showed expressional discrepancy in petiole.

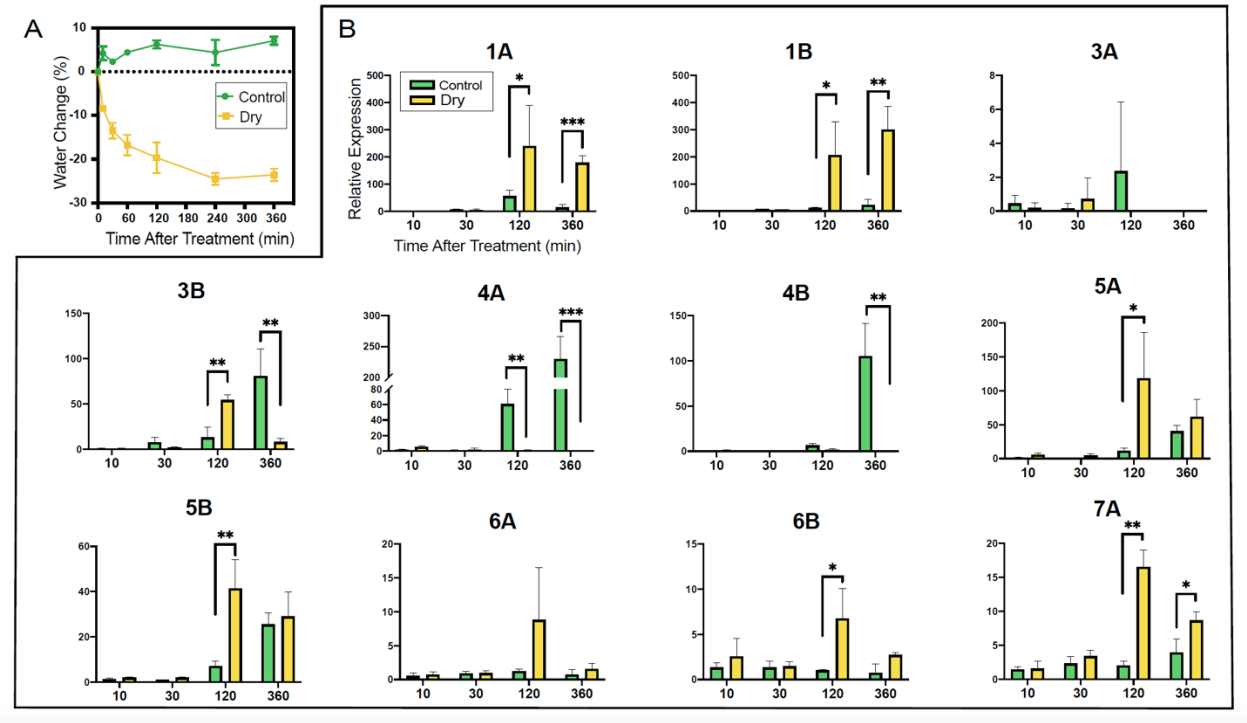

Figure 15. A subset of apple *GA2ox* genes were induced by water loss within two hours in leaves. The first fully expanded leaves were exercised from rapidly growing seedlings and either left on a bench to air dry (Dry, in yellow) or kept hydrated with the tip of petiole touching water (control, in green). A. Changes of water content in the control (green line) and air-dry leaves (yellow line). The water content remained relatively stable over time in the hydrated leaves, whereas that in air-dry leaves decreased dramatically and reached its lowest point as soon as 4 hours after treatment. Leaf water change is defined as the difference between the after-treatment weight and the initial weight: Water Change% = (Second Weight - Original Weight)/Original Weight*100%. B. Relative gene expression in the control (green bar) and air-dry (yellow bar) leaves over time. Expression was detected at 10 min, 30 min, 120 min (2 h) and 360 min (6 h) after treatment.

Among 14 genes studied, only five of them showed response to salt treatment in the young leaf. Specifically, *MdGA2ox1A* and *-1B* were induced as early as 10 min after treatment; *MdGA2ox6B* was downregulated at 10 min but induced at 30 min and 6 h after treatment. None of these three genes were differentially expressed at later time points (S3 Fig.). Unlike *MdGA2ox1A*, -1B or -6B, *MdGA2ox4A* and *-4B* were significantly downregulated only at later time points, for instance, at 2 d or 6 d after treatment (S3 Fig.). There are two stages of salt stress response in plants (Taiz et al. 2015). The first stage

involves response to osmotic pressure where plants sense the difference of osmotic pressure caused by salt stress and start to lose water. This happens fast and is actually water deficit stress resulting from salt stress. The second stage is the response to ion toxicity where ions build up in leaves and eventually burn or deprive leaves of function. The second stage is considered to happen much later. According to our observations, it is likely that the differential expression of *MdGA2ox1A*, *-1B* and *-6B* was credited to the initial water loss caused by salt treatment instead of salt ion toxicity (e.g., Na^+ or Cl^-). However, the downregulation of *MdGA2ox4A* and *-4B* might be due to either staged response.

Clustering and correlation analysis

As duplicated genes may have retained certain *cis*-regulatory elements and/or functions during evolution, it would not be surprising if duplicates share similar expression profiles and overlapping function. In this study we found that the apple *GA2ox* genes are likely eight pairs of duplicated genes (Fig. 9B). A clustering analysis of gene expression showed that most pairs (except for *MdGA2ox7A*/*-7B* and *MdGA2ox3A*/*-3B*) were expressed in similar patterns to their homoeologs (Fig. 16A), which future supports that they are duplicated gene pairs. Expression of *MdGA2ox5A* and 5B was highly positively correlated (Fig. 16B). Expression of *MdGA2ox4A*/*-4B* was similar to each other but very distinct from the rest of genes. However, the fact that some pairs were expressed similarly in certain structures but not in other structures suggests that the function of these pairs may be partially conserved.

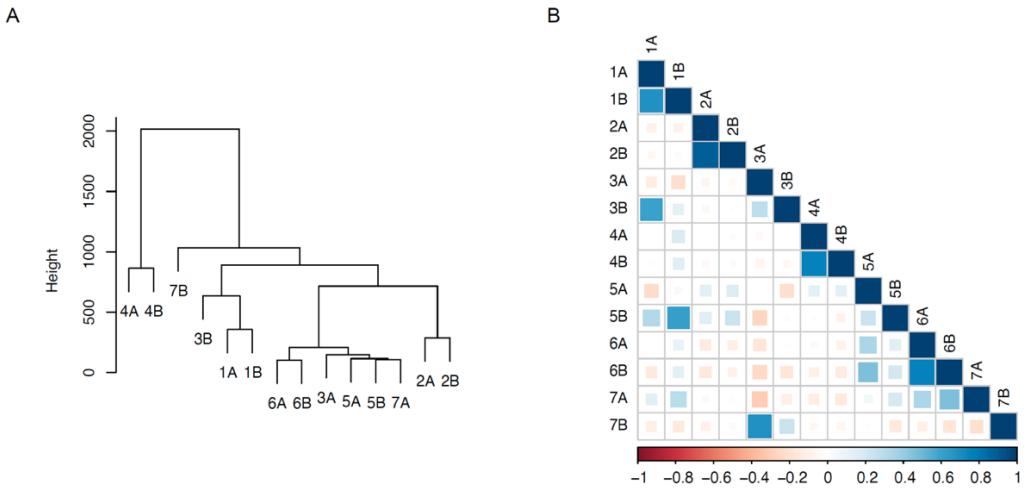

Figure 16. Clustering and correlation analysis of apple *GA2ox* genes. A. The dendrogram depicts the relationship among apple *GA2ox* based on their expression. The y axis represents 'Height' or Euclidean distance. B. Expression correlation. The heat map represents the correlation values between two individuals (dark blue, strongly positively correlated; dark red, strongly negatively correlated).

Discussion

Retention of the apple *GA2ox* genes

Using the newly available apple reference genomes 'GDDH13' (Daccord et al. 2017), the 'Gala' phased diploid genome (Sun et al. 2020) and our transcriptome datasets derived from the 'Gala' shoot apex (Zhang et al. 2019), we carried out a rigorous genome-wide census of *GA2ox*-like genes and identified a total of 16 canonical ones in the apple genome (Fig. 9), in contrast to 10 genes in Arabidopsis (Rieu et al. 2008; Lange et al. 2020), 13 in *Zea maize* (Li et al. 2021), 11 in tomato (Chen et al. 2016), 11 in grape (He et al. 2019), 11 in poplar (Gou et al. 2011), 7 in peach (Cheng et al. 2021). A further examination of their genomic organization revealed that these 16 genes are likely eight pairs of duplicated genes (Fig. 9B). Retainment of duplicated *GA2ox* gene pairs have been reported in many other flowering plant species including *Zea maize* (Li et al. 2021), grape (He et al. 2019), poplar (Gou et al. 2011) and peach (Cheng et al. 2021). However,

the apple *GA2ox*-like genes are relatively over-retained during evolution (16 genes = 8 homoeologous pairs) compared to other closely related plant species. Both whole genome duplication events and smaller-scale duplications such as single-gene duplication can result in gene duplicates. In any case, duplicated genes are either retained or, more frequently, lost during evolution (Reviewed in Edger and Pires 2009). This process is considered to have played an important role in facilitating speciation and improving morphological and developmental complexity. It is not clear how and when the *MdGA2ox* genes were duplicated, but their over-retention in the apple genome may have enabled more diverse GA activities in apple.

We found that all 16 *MdGA2ox* loci can potentially produce a protein with two characteristic domains and conserved residues (Fig. 10). Using highly sensitive and specific Taqman primer and probe sets, we found that each of the 14 loci examined here is expressed in several structures, suggesting their potential biological significance (Figs. 12 and 13). However, expression and presence of active sites in predicted protein structure do not guarantee a functional product, as regulation at post-transcriptional, translational, and post-translational levels may exist and affect its final function. Therefore, the enzymatic function and activity remains to be studied.

Expression, function divergence and redundancy

Multiple *GA2ox* members may allow for precise GA homeostasis in response to various cues. Where studied, specific *GA2ox* genes have been shown to be functionally redundant. For instance, knockout of individual genes in Class I and Class II failed to lead to visible phenotypes in Arabidopsis (Rieu et al. 2008). Meanwhile, the diversity in spatial and temporal expression of *GA2ox* genes suggests potential uniqueness in function,

especially in the context of response to environmental stress or phytohormones. For example, *AtGA2ox2* and AtGA2ox4 are both expressed at the base of the shoot apical meristem, but only *AtGA2ox2* is inducible by cytokinin (Jasinski et al. 2005). In addition, *GA2ox* genes are induced in response to abiotic stresses such as drought, cold and salt, and touch (reviewed by Colebrook et al. 2014; Lange and Lange 2015), presumably to arrest new growth (Hedden 2020). The regulatory network involving *GA2ox* genes has been expanding the past two decades and is expected to continue to expand.

By examining the expression profiles of apple *MdGA2ox* genes in both spurs and seedlings under various conditions, we observed that genes are both spatially and developmentally regulated (Figs. 11-15), which is not surprising and has been reported in other plant species. However, using correlation and clustering analysis based on our existent expression data, we found that expression of most of the homoeologous pairs was highly correlated, indicating potential functional redundancy in specific structures (Fig. 16). However, occasional discrepancy in expression in other structures suggests function divergence. We speculate that the large number of gene copies along with the vast web of spatial and developmental expression is critical for fine tuning GA levels and activity in apple.

MdGA2ox in floral induction

We previously found that a small subset of *MdGA2ox* genes (namely, *MdGA2ox1A*, *-1B*, *-2A* and *-2B*) were induced by both exogenous GA and the presence of fruits (or crop load) in the shoot apex during the anticipated period of floral induction, suggesting that they may be involved in GA's repression of apple flowering (Zhang et al. 2019; Gottschalk et al. 2021). It is consistent with a previous study where *MdGA2ox1A* and *MdGA2ox1B*

were induced by the presence of fruits in 'Gala' during the period of floral induction (at 48 and 119 days after full bloom) (Guitton et al. 2016). In the present study, we found that even though *MdGA2ox1A*/*-1B* were not preferably expressed in the shoot apex during the period of floral induction, they were strongly GA-responsive in this structure (Figs. 11 and 13). In contrast, *MdGA2ox2A*/*-2B* were both predominantly expressed in the shoot apex while being highly GA-responsive (Figs. 11 and 13). Collectively, these results indicate these four *MdGA2ox* genes are involved in regulating GA's activity during floral duction.

Expression in response to environmental cues

In addition, we found that a subset of genes can be quickly induced by acute water deficit and salt stresses (Fig. 15, S2 and S3 Figs.), which is consistent with previous findings in Arabidopsis (Achard et al. 2008; Magome et al. 2008). It is believed that under certain stresses such as cold, drought and salt, plants usually strive to restrict growth by reducing endogenous bioactive GA levels, and the immediate breakdown of bioactive GAs is at least partially through regulation of *GA2ox* genes (reviewed in Colebrook et al. 2014). Indeed, overexpression of a *GA2ox* gene in poplar resulted in enhanced tolerance to drought (Zawaski and Busov 2014). We suspect that their active involvement in response to various abiotic stresses may have contributed to their over-retention in evolution.

In summary, to advance our understanding of *GA2ox* genes in the apple genome and to facilitate the breeding programs harnessing these genes, we explored their copy number and genomic organization as well as the spatial and developmental expression. We found that the apple genome contains at least 16 copies of *MdGA2ox* and that they

represent eight pairs of duplicated genes. Their spatial and developmental expression will provide hidden information of their function and guide manipulation of *MdGA2ox* genes for breeding purposes. Further characterization of *MdGA2ox* and the regulation of *MdGA2ox* could lead to more specific manipulation of GA-related traits (Busov et al. 2003). Due to the ability of the GA 2-ODDs to control GA concentration, there have been increasing research studies on GA metabolic genes in perennial woody plants, generally with the goal to develop trees with desirable GA-associated traits such as tree stature, flowering time and improved abiotic stress tolerance by genetically manipulating these genes (El-Sharkawy et al. 2012; Gou et al. 2011; Zawaski and Busov 2014; Bulley et al. 2005; He et al. 2019; Cheng et al. 2021; Zawaski et al. 2011). For woody trees, GA deficiency is usually desired for dwarf/semi-dwarf tree stature and enhanced abiotic stress tolerance. However, risks exist in manipulating a random GA metabolic gene without a comprehensive understanding of the gene family, as low GA levels can not only introduce desired GA-associated traits but also the undesired into transgenic trees such as defects in flower and fruit development, and low fruit set (Bulley et al. 2005; Zawaski et al. 2011), any of which could be a deal breaker in breeding. Even though these risks may not be as significant in rootstock development, where decreased GA levels could result in dwarf/semi-dwarf rootstocks with improved tolerance to stress but may not necessarily affect fruit development in scions, a better understanding of the function, spatial and developmental expression of the gene family is still both necessary and important. Decreased GA levels can be effectively achieved by knocking down/out GA biosynthetic genes such as *GA20ox* and *GA3ox* or overexpressing *GA2ox* genes. However, partial function redundancy exists in each gene family (Mitchum et al. 2006;

Rieu et al. 2008), knock-out/-down of a single *GA20ox* or *GA3ox* gene may or may not be as effective or precise as site-specific overexpression of a single *GA2ox* gene. A future direction would be modification of *MdGA2ox* in rootstocks, for dwarf/semi-dwarf tree stature, efficient water and nutrient absorption and enhanced tolerance to abiotic stresses, rather than directly in scions.

Methods

Genome-wide census of apple *GA2ox* genes

Homology-based identification of *MdGA2ox* genes among all genes previously annotated in the GDDH13 reference genome used the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al. 1990) and the GDDH13 protein (https://iris.angers.inra.fr/gddh13/downloads/GDDH13_1-1_prot.fasta), or mRNA (https://iris.angers.inra.fr/gddh13/downloads/GDDH13_1-1_mrna.fasta) reference datasets. The query sequences comprised all 14 peptide sequences cataloged as "Gibberellin 2-beta-dioxygenase" in ExPASy (https://enzyme.expasy.org/EC/1.14.11.13): 7 GA2ox sequences from Arabidopsis, 4 from rice, 2 from pea, and 1 from bean (UniProtKB/Swiss-Prot accessions: G2OX1_ARATH, Q8LEA2; G2OX2_ARATH, Q9XFR9; G2OX3_ARATH, O64692; G2OX4_ARATH, Q9C7Z1; G2OX6_ARATH, Q9FZ21; G2OX7_ARATH, Q9C6I4; G2OX8_ARATH, O49561; G2OX1_ORYSJ, Q5W726; G2OX2_ORYSJ, Q5ZA21; G2OX3_ORYSJ, Q8S0S6; G2OX6_ORYSJ, Q7XP65; G2OX1_PEA, Q9SQ80; G2OX2_PEA, Q9XHM5; G2OX_PHACN, Q9XG83) (**Appendix S1**). At an Expect (e)-value cutoff of 1E-12, this resulted in the identification of 185 GDDH13 gene names. The designated open reading frame translation from each gene model was retrieved from the GDDH13 protein database

(https://iris.angers.inra.fr/gddh13/downloads/GDDH13_1-1_prot.fasta) (S2 file) and used as a query to reciprocally interrogate a dataset of Arabidopsis open reading frame translations (TAIR10_pep_20101214; TAIR10; http://arabidopsis.org) with e<1E-12. This resulted in 118 Arabidopsis protein names (S3 file).

HMMer (http://hmmer.org) was implemented with hmmbuild to create a profile based on the multiple alignment of the 14 GA2ox index sequences, which was then used as queries with hmmsearch and an E-value of 0.01 for whole sequence and the best match domain, this approach resulted in 233 apple genes. Protein sequences of these 233 homologs were retrieved from the reference genome (GDDH13_1-1_prot.fasta) using shell scripts. These sequences were scanned for domains in the Pfam database (El-Gebali et al. 2019, https://pfam.xfam.org).

As HMMer analysis did not result in identification of additional homologs, only the first set of sequences along with 14 query sequences were aligned separately using MUSCLE and then analyzed for phylogenetic relationship using MEGAX (version 10.1.8) with the phylogenetic trees shown in Figure 9A generated using the maximum likelihood method and a bootstrap of 1000 replicates. All positions with less than 80% site coverage were eliminated (partial deletion option). There was a total of 306 positions in the final dataset.

Gene, transcript, and protein model analysis

Gene models were mainly based on the apple reference genome ‘GDDH13’ (Daccord et al. 2017) and curated with the ‘Gala’ phased diploid genome (Sun et al. 2020); http://bioinfo.bti.cornell.edu/apple_genome/) and transcriptome datasets derived from the ‘Gala’ shoot apex (Zhang et al. 2019). Gene models were visualized using IGV_2.8.13

and SnapGene (version 5.3.2). As the ‘Gala’ reference genome is more consistent with our transcriptome datasets in general, we used the sequences of the consensus gene models for protein structure analyses and to build a refined phylogenetic tree (Fig. 9B). Loci with inconsistent gene models were curated based on the reads from the transcriptome datasets. Putative amino acid sequences were predicted based on the open reading frame sequences using ORF Finder (https://www.bioinformatics.org/sms2/orf_find.html). Protein structures from novel transcripts and curated gene models were analyzed using SMART (Simple Molecular Architecture Research Tool, http://smart.embl-heidelberg.de/).

Plant materials and growth conditions

‘Gala’ apple trees (‘Brookfield Gala’ grafted on ‘M9-Pajam 2’ rootstocks) used to detect the expression of *MdGA2ox* genes in response to GA and in different spur structures were located at the Michigan State University Research Center in Clarksville, MI (Field: 42°52'24"N, 85°16'05"W – Station: 42°52'24"N, 85°15'30"W) and were managed in accordance with standard commercial practices for disease, insect and weed control. The date of full bloom was defined as the date on which the maximum numbers of flowers were at anthesis. Three trees were sprayed with GA_3 at a concentration of 400 parts per million (ppm) at 30 days after full bloom (DAFB); three control trees were sprayed with water; each tree served as a biological replicate. Regulaid (0.1%) was used as surfactant for each spray. For gene expression in spur structures, there were three biological replicates with each consisting of 15 spurs from three trees (5 spurs/tree). Fruiting spurs were collected from nine normally cropping trees, and non-fruiting spurs from nine naturally under-cropping trees at 40 DAFB in 2021 (due to frost damage in

2019). Fruiting spurs were then dissected into 11 substructures including shoot apex, bourse leaf, petiole of the bourse leaf, base of bourse leaf petiole, spur leaf, petiole of spur leaf, base of spur leaf petiole, pedicel, base of pedicel, immature seeds, and fruit (longitudinally cut with skin, ~3 mm thick). For each spur, only the biggest fruit on each spur was collected for dissection. Transverse sections (~2 mm thick) of the fully expanded bourse leaf and spur leaf that were most adjacent to the shoot apex and the biggest fruit, respectively, were dissected. Non-fruiting spurs were dissected into four substructures, including shoot apex, bourse leaf, petiole of bourse leaf and base of bourse leaf petiole. Samples were collected into liquid nitrogen immediately following dissection at 40 DAFB and stored at -80°C until use. Embryos, endosperms, and seed coats were dissected from about 150 immature seeds per biological replicate at 37 DAFB in 2019. Three trees served as three biological replicates. For seed dissection, about 50 fruiting spurs were exercised from each tree, wrapped with wet paper towels stored in loosely sealed plastic bags, and transported back to the laboratory where three healthy seeds were then extracted from each fruit and substructures were dissected directly into liquid nitrogen.

For experiments with seedlings, seeds were obtained from fruits of open-pollinated ‘Gala’ trees and subjected to stratification at 4°C for three weeks in moist vermiculite (fruits were pre-stored in the cold for months). Germinating seeds were transferred into square pots (size: 10 cm*10 cm*10 cm) containing artificial soil mix (Sungro Professional Growing Mix). Plants were maintained in a controlled environment chamber under 16h-light/8h-dark photoperiods. Lighting was supplied with white fluorescent lights (Philips 32-watt, model: F32T8/TL741, average light intensity: 154 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The temperature was held at 25°C during the light period and 18°C during the dark period. The relative

humidity ranged from 25% to 28% in both the light and dark periods. Experiments to evaluate gene expression in various seedling structures, as well as diurnal expression, and changes in expression in response to water loss and salt exposure were carried out three weeks after transfer, when plants had developed 9-11 true leaves and were ~20 cm tall. For analysis of developmental expression, structures were excised using a razor blade between 5 h and 7 h diurnal time. To evaluate diurnal changes in expression, the first fully expanded leaf and 3-5 mm shoot apex were collected. Each sample comprised pooled tissue from 10-15 plants, and all experiments utilized three biological replicates sampled within 5 min. Dissected tissue was immediately frozen in liquid N_2.

Leaf water loss experiment

To evaluate gene expression in the leaf in response to water loss, the first fully expanded leaf was excised at the base of petioles using a razor blade and placed on a bench at ambient conditions. Specifically, a total of 54 plants were randomly assigned into two groups (air dry and control). Each group consisted of eight subgroups. Each subgroup had three replicates with each replicate consisting of three first fully expanded leaves with petioles cut from three individual plants. The first fully expanded leaves were cut 2 mm from the base of the petiole and weighed immediately following the cut. The “air dry” group of leaves were left on the bench in the same growth room with no change in the environmental conditions. For control, leaves were kept hydrated by putting the petioles in water. Eight subgroups of leaves in both “air dry” and control were weighed again and cut 2 mm from the base of petioles again. Leaf blades and petioles were collected separately into liquid nitrogen for RNA extraction at 10 min, 30 min, 2 h, and 6 h, respectively. Treatment time in the experiment was between 12 pm and 1 pm

corresponding to 5 h and 6 h diurnal time, respectively. Sample collection was conducted between 12:10 pm and 7 pm which were 5.17 h and 12 h diurnal time, respectively.

Salt treatment

To examine the expression of *MdGA2ox* genes under salt, we treated rapidly growing apple seedlings with 100 mM salt (NaCl) and detected their expression at several time points after the treatment. Specifically, over 500 young seedlings were well watered and fertilized two days before the experiment. About 2 hours prior to treatments, the plants were watered accordingly to make sure that the soil in each pot was fully saturated with water. Excessive water in the bottom tray was drained before treatments. The plants were either treated with 100 mL of 100 mM salt or 100 mL of water (control). Since the absorption rate of the soil may vary, the salt solution or water was gently poured from the top of the pots. The excessive salt solution or water in the bottom tray was drained immediately following the treatment. Young leaves with a diameter of 0.5-1 cm (one leaf per seedling) were excised at 10 min, 30 min, 2 h, 6 h, 1 d, 2 d, and 6 d after treatment, respectively, and quickly collected into liquid nitrogen. Sample collection in this experiment was conducted between 11:30 am and 6:30 pm which were 4.5 h and 11.5 h diurnal time, respectively. There were three biological replicates at each time point in two treatment groups (salt or control) with each replicate containing 10-15 young leaves.

RNA extraction and RT-PCR

Total RNA was extracted using the method of (Gasic et al. 2004), with the exception that spermine was substituted for spermidine in the extraction buffer, followed by a final 'clean-up' step using a commercial kit (RNeasy Mini; Qiagen). RNA was treated with DNase I to remove genomic DNA. A small amount of RNA was checked on Nanodrop

and an FAA agarose gel for quantity, quality, and integrity. About 1 ug of RNA was then reversed to cDNA (High-Capacity Reverse Transcriptase Kit, ThermoFisher). RNA-seq and sequence assembly are documented in (Zhang et al. 2019). Taqman primer and probe sets were designed according to the curated *MdGA2ox* transcript sequences in our 'Gala' dataset (Fig. 10 and S4 Table). The primer/probe design rules and quantitative RT-PCR (Real Time-Polymerase Chain Reaction) have been described in detail in our previous study (Zhang et al. 2019). For primer and probe sets that generate primer dimer(s) detected in the no-template control (e.g., 1A, 1B and 2A), Ct thresholds were adjusted to surpass the amplification of primer dimer(s).

Clustering and correlation analysis

Clustering and correlation analysis was conducted in RStudio (version 1.1.463) using packages *Hmisc* and *corrplot*, respectively. Euclidean distance followed by implementation of the *Ward 2* algorithm was used for clustering, while Pearson correlation coefficients were used for correlation analysis.

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CHAPTER FOUR

A Genetic Tool Kit to Study *GA2ox* Gene Function in *Arabidopsis thaliana*

ABSTRACT

Catabolism of gibberellins (GAs) by GA2-oxidases (GA2ox) is the predominant pathway for GA inactivation and an important component of GA homeostasis. Expression of *GA2ox* genes is under tight regulation in response to endogenous and environmental cues. Even though their general and overlapping roles in plant growth and development have been studied, their specific functions are not fully understood. To study their functions, we developed a genetic tool kit comprising both CRISPR/Cas-induced, transgene-free single knockout mutants and transcriptional and translational GUS fusion lines for the seven canonical Arabidopsis *GA2ox* genes (*AtGA2ox1-AtGA2ox8*). Mutations in the loss-of-function mutants have presumably disrupted function of both two functional domains of the GA2ox proteins. By comparing GUS activity in the GUS fusion lines, we found that the transcribed region is required for appropriate expression for several genes. Using this tool kit, we observed multiple GA-associated phenotypes in the *ga2ox2* mutants that resembled the phenotypes reported in a *ga2ox* quintuple mutant (*ga2ox1*/-2/-3/-4/-6), including defects in flower development, increased number of flowers and siliques, and seed abortion. We also found GA-deficient phenotypes in the *GA2ox2*-GUS translational lines expressing two additional copies of *GA2ox2* under its native promoter. By comparing opposite phenotypes between the loss-of-function mutants and GUS translational lines, we suspect that *AtGA2ox2* might be responsible for multiple GA-associated traits. This tool kit can be used for a wide spectrum of GA biology.

Introduction

Gibberellins (GAs) are synthesized in multiple steps through several groups of enzymes (Hedden and Thomas 2012). The first step is the production of *ent*-kaurene from trans-geranylgeranyl diphosphate (GGPP) by two diterpene cyclases, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). Sequential oxidation by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO) converts *ent*-kaurene into the initial GA form in the pathway, GA_{12}. The later steps involve sequential oxidation of non-bioactive C-20 GA precursors into active C-19 GAs by two groups of 2-oxoglutarate-dependent dioxygenases (2-ODDs), GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox).

In *Arabidopsis thaliana* (Arabidopsis), enzymes in the first two steps (CPS, KS and KO), except for KAO, are encoded by single-copy genes. Mutations in any of these genes display the most severe GA-deficient phenotypes such as severe dwarfism and dark green leaves (Koornneef and van der Veen 1980). In Arabidopsis, KAO is encoded by two partially functional redundant genes, *KAO1* and *KAO2*. Single knockout of either *KAO* gene did not confer apparent phenotypes, however, the *kao1 kao2* double mutant showed characteristic GA-deficient phenotypes (Regnault et al. 2014). GA20ox and GA3ox are encoded by multigene families. The Arabidopsis genome comprises five and four copies of *GA20ox* and *GA3ox*, respectively. *GA20ox1*, *GA20ox2* and *GA20ox3* are the dominant *GA20ox* genes, as the function loss of these genes led to the most severe dwarfism and infertility, compared with wild-type and their single and double mutants (Plackett et al. 2012). Despite that, three genes showed partial functional redundancy with one another (Rieu et al. 2008; Plackett et al. 2012). Similarly, mutant analyses suggest that two *GA3ox*

genes, *GA3ox1* and *GA3ox2*, also have distinct and overlapping roles (Mitchum et al. 2006). Due to functional redundancy, the single mutants of *GA20ox* and *GA3ox* (e.g. *ga4* and *ga5*) showed no or less severe GA-deficient phenotypes (Koornneef and van der Veen 1980; Talon et al. 1990).

An additional group of 2-ODD enzymes, GA2-oxidases (GA2ox), catalyze 2β-hydroxylation of both bioactive and inactive GAs, and GA2ox catabolism is considered as the major GA inactivation pathway (Rieu et al. 2008). The Arabidopsis genome contains nine *GA2ox*-encoding genes (*GA2ox1*-*GA2ox10*; *GA2ox5* is a pseudogene) (Thomas and Hedden 1999; Hedden and Phillips 2000; Schomburg et al. 2003; Lange et al. 2020). GA2ox enzymes comprise three distinct classes (Lee and Zeevaart 2005; Serrani et al. 2007). Class I and II GA2oxs specifically target C19-GAs and are capable of deactivating bioactive GAs. In Arabidopsis, Class I enzymes include GA2ox1, GA2ox2, and GA2ox3, whereas Class II enzymes include GA2ox4 and GA2ox6 (Table 1). Distinction between Class I and Class II enzymes is based on amino acid sequence and catalytic activity. Class I members (except GA2ox1) have multi-catalytic activity and can produce GA catabolites by further oxidation, whereas Class II members have mono-catalytic activity and so are not capable of producing GA catabolites (Thomas and Hedden 1999; Serrani et al. 2007). Class III comprises two additional GA2ox proteins, GA2ox7 and GA2ox8, which were shown to use C20-GAs (GA_{12} and GA_{53}), but not C19-GAs, as substrates (Schomburg et al. 2003) (Table 1). Based on this, they are likely to be involved in regulating early intermediates but not the degradation of the active GAs. Recently, two more Class III GA2ox proteins, GA2ox9 and GA2ox10, were characterized (Lange et al.

2020). Interestingly, it is shown that GA2ox9 can use C19-GAs as substrates (Lange et al. 2020).

Like *GA20ox* and *GA3ox*, this group of genes are expressed throughout the Arabidopsis's life cycle, and their function is both distinct and redundant to a certain degree. Both transcriptomic and targeted approaches have resulted in preliminary characterization of the spatial and temporal expression of the *GA2ox* genes, both under ideal growth conditions (S6 file) and in the presence of abiotic and biotic stress. Expression profiling of C19-*GA2ox* genes (genes in the first two Classes) in Arabidopsis using RT-PCR revealed *AtGA2ox2* and *AtGA2ox6* are the most highly expressed genes in all the structures studied by Rieu et al. (2008). Even though they had distinct expression profiles, single knockout of each gene failed to confer any apparent phenotype likely due to their widespread co-expression throughout development (Rieu et al. 2008). Knockout of all five C19-*GA2ox* genes (*GA2ox1*, *-2*, *-3*, *-4*, and *-6*) resulted in severely reduced levels of their 2b-hydroxylated products and enhanced levels of bioactive GAs. In addition, the *ga2ox1 ga2ox2 ga2ox3 ga2ox4 ga2ox6 quintuple* mutant showed high seed germination rate without cold treatment or in darkness, increased number of flowers per inflorescence, elongated pistils and infertility, Class III members, *AtGA2ox7* and *AtGA2ox8* share higher homology with each other than with other genes. Overexpression of either gene led to severe dwarfism (Schomburg et al. 2003). Recently, a pair of new Class III GA2ox enzymes, AtGA2ox9 and AtGA2ox10, were characterized in Arabidopsis (Lange et al. 2020). Single knockout mutants of *GA2ox9* and *GA2ox10* showed elongated hypocotyls. *GA2ox10* may have a role in regulating fertility, as an increased number of seeds per silique was observed in the *ga2ox10* mutants; *GA2ox9* may contribute to

freezing tolerance, as loss of *GA2ox9* conferred hyposensitivity to freezing (Lange et al. 2020).

The currently available *ga2ox* loss-of-function mutants are mostly T-DNA insertion lines, which thus are transgenic (S7 file). The insertion sites reside across the promoter, both un-transcribed and transcribed regions. For several genes (*GA2ox4*, *GA2ox6* and *GA2ox8*), however, T-DNA insertions reside either within introns or the last two exons, and thus does not interrupt the function of the N-terminus domain (DIOX_N domain) (S7 file), making it tricky to select an effective loss-of-function line. In the present study, I developed CRISPR/Cas9-mediated single knockout mutants that are transgene-free and contain biallelic homozygous mutations within the sequence that encodes the conserved domain DIOX_N. *GA2ox-GUS* fusion lines have been developed for expression analyses in a higher resolution than RT-PCR (Jasinski et al. 2005; Bolduc and Hake 2009; Li et al. 2019). As previous studies showed that introns are required for proper expression of two GA biosynthetic genes (Silverstone et al. 1997; Itoh et al. 1999), both the promoter and the transcribed region are included in the GA2ox-GUS fusion lines. Comparisons of GUS activity in several *GA3ox* and *GA20ox* transcriptional (promoter only) and translational (promoter plus the transcribed region) with expression data obtained from RT-PCR indicated that the transcribed region is either not required or only required in specific developmental stages for these genes, and that GUS activity transcriptional lines was correlated more with RT-PCR data in most cases (Mitchum et al. 2006; Plackett et al. 2012). However, it is unknown if the promoter alone is sufficient or if the transcribed region is indeed required for the proper expression of *GA2ox* genes. To answer this question, I developed both transcriptional and translational GUS-fusion lines for each of the

Arabidopsis *GA2ox* genes (excluding the newly characterized *GA2ox9* and *GA2ox10*) in the present study. The newly developed GUS-fusion lines and transgene-free single knockout mutants can be used together as a toolkit in various contexts including *GA2ox* gene regulation in response to internal and external cues, regulation by cis-regulatory element-binding transcription factors, and gene function. Herein, the *GA2ox2-GUS* fusion lines and the *ga2ox2* mutants were used for further function analyses.

Results

Development of a panel of CRISPR-edited *GA2ox* genes

To generate effective loss-of-function mutations, we firstly analyzed the locations of the conserved regions of the Arabidopsis *GA2ox* (Fig. 17 and S8 file). We developed a CRISPR/Cas9 gene editing construct containing a pair of crispr RNAs (crRNAs) with the first crRNA targeting the DIOX_N-coding region (Figs. 17 and 18). To avoid mosaic editing, the *Zea mays* codon-optimized *zCas9* gene was driven by an egg cell-specific promoter (*AtE.C1.1p*) (Fig. 18). A depiction of the *GA2ox* genes, showing the gene models, the locations of two major domains, crRNAs and detection primer as well as sites for diagnostic restriction is shown in Figure 17. This approach resulted in 17 independent single knockout mutants at seven *GA2ox* loci, including one at the *GA2ox1* and *GA2ox4* loci, two at the *GA2ox6* locus, three at the *GA2ox2*, *GA2ox3* and *GA2ox8* loci, and four at the *GA2ox7* locus (Fig. 19). All these lines contained mutations that presumably lead to immediate premature stop codons (data not shown). Mutations were both confirmed with sequencing and detection markers (Fig. 17). This specific CRISPR/Cas gene editing system induced relatively small indels (1 - 27 bp) primarily located at the first crRNA-

targeting sites (Fig. 19), and the editing efficiency at the second crRNA sites was compromised due to unknown reason.

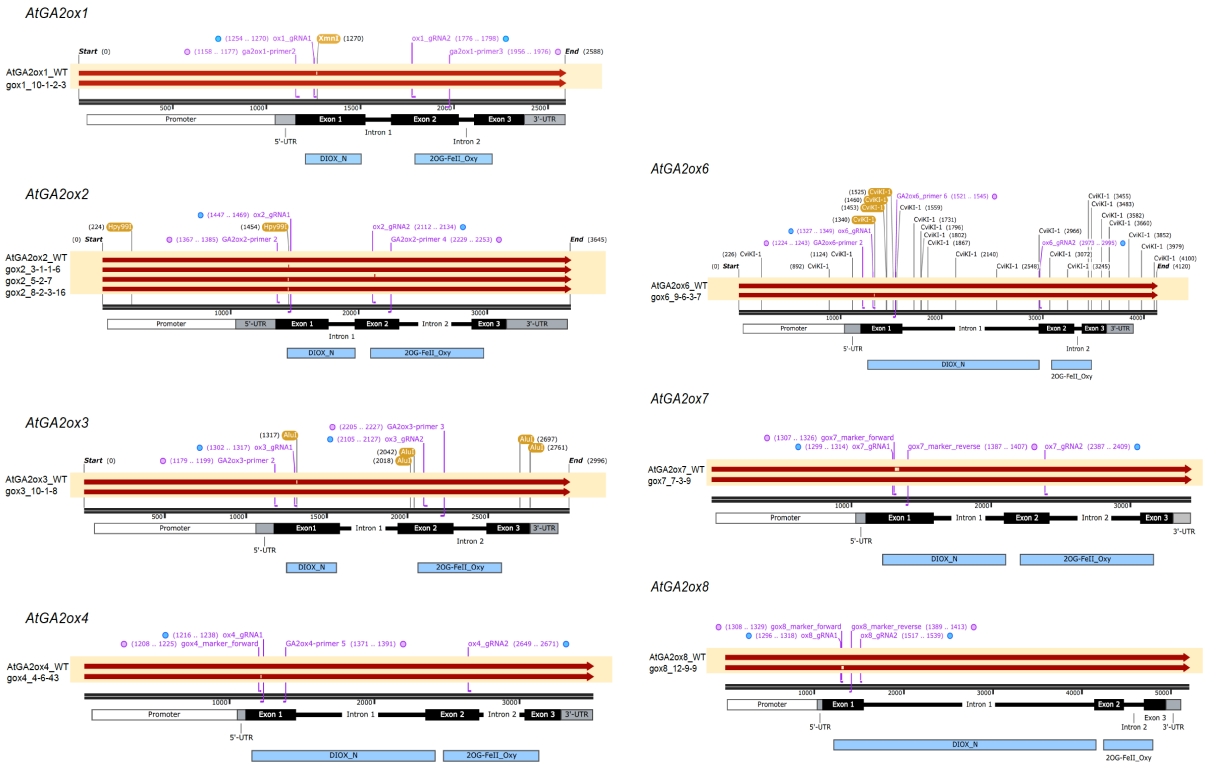

Figure 17. Illustration of the Arabidopsis *GA2ox* gene models with details of CRISPR/Cas9-induced mutations. For each locus, a representative gene model was given, where gene structures are depicted with white (promoter), black (exon), grey (untranslated regions or UTRs) boxes and thick black solid lines (intron). A scale (unit bp) is shown above the gene model. Two major functional domains (DIOX_N and 2OG-FeII_Oxy) are shown with blue boxes, which are below the enxon regions (excluding introns) that encode the corresponding domains. The wild-type *GA2ox* sequence was aligned with the representative mutant(s), which are shown in red. The locations of two crRNA-targeting sites (ox_gRNA1 and ox_gRNA2, highlighted in blue) were relative to the gene model and the mutant(s). Primers and restriction enzymes for mutation detection were highlighted in purple and golden. Where restriction enzymes were designed, sequence(s) without gaps or other symbols can be cut by this specific enzyme at the mutations.

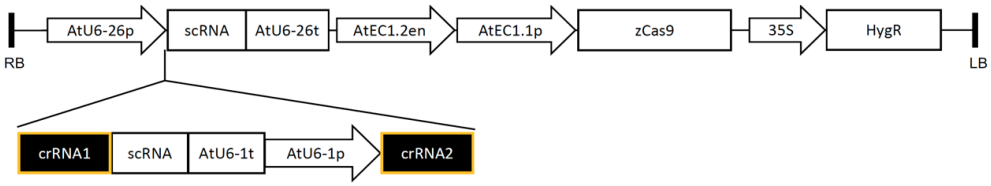

Figure 18. Illustration of the CRISPR/Cas9 knockout construct. Two crisprRNAs (crRNA1 and crRNA2), along with a copy of scaffoldRNA (scRNA) and a copy of Arabidopsis U6-1 promoter and terminator, were amplified and inserted into the pHEE401E vector downstream of the Arabidopsis U6-26 promoter and upstream of a scRNA. Details of vector pHEE401E can be found in Wang et al. 2015. Locations of the crRNA-targeting sites specific to each locus can be found in Figure 17.

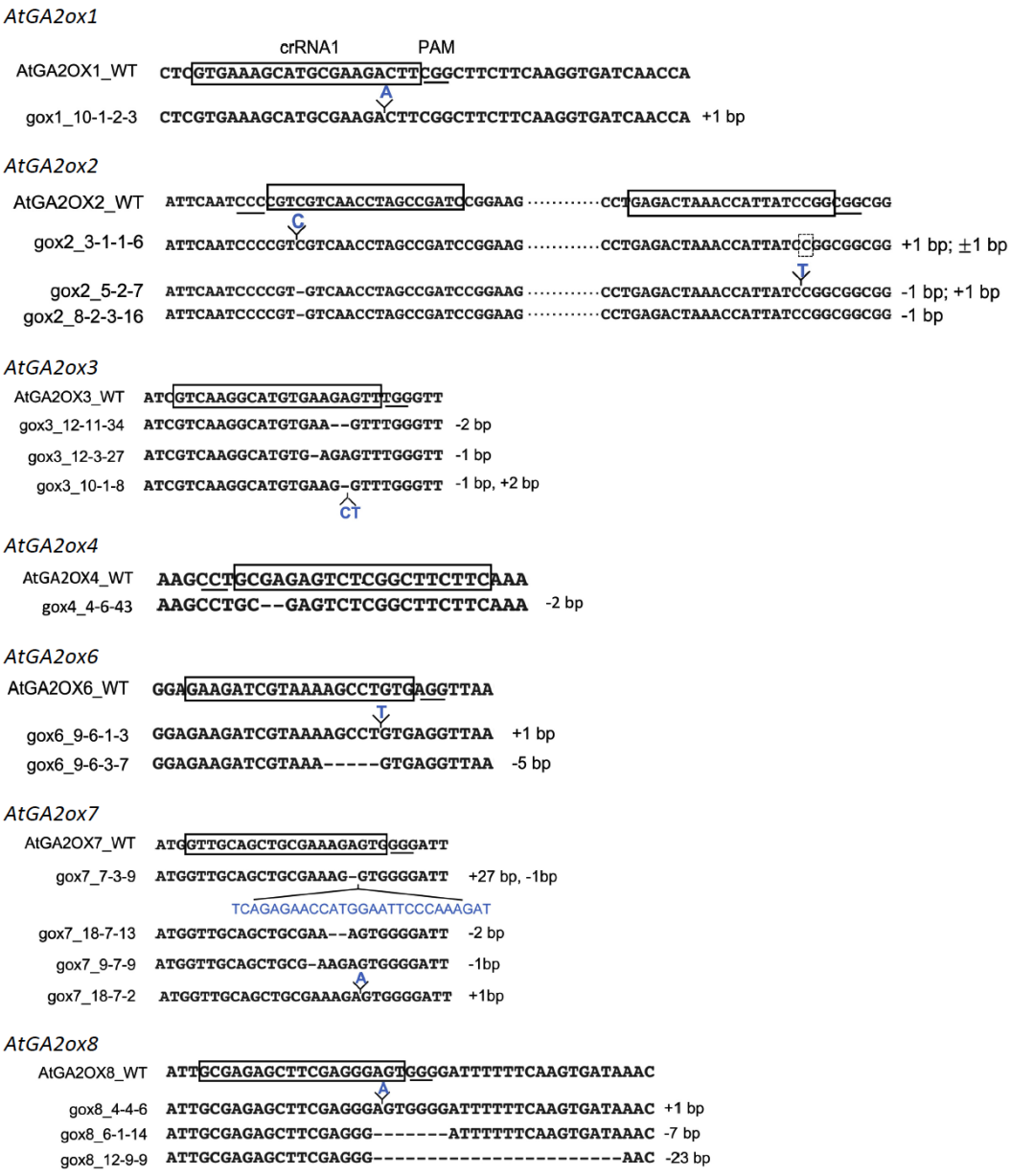

Figure 19. CRISPR/Cas9-induced mutations and the *AtGA2ox* loci. For each locus, the crRNA recognition site is highlighted in a box and the PAM (proto-spacer adjacent motif) sequence (5'-NGG) is underlined. Sequences highlighted in blue represent inserted sequences in mutants, dashed lines (--) represent deleted sequences compared with the wild type. Dashed box denotes heterozygosity at this location (1-bp deletion in one allele and 1-bp insertion in another).

Phenotypes of CRISPR/Cas-induced *ga2ox* mutants

GA promotes Arabidopsis seed germination, stimulates hypocotyl and stem elongation, and promotes flowering in both long and short days (Karssen et al. 1989; Cowling and Harberd 1999; Langridge 1957; Wilson et al. 1992). To determine whether the knockout of individual genes leads to any apparent GA-associated phenotypes, 7-d seedlings and 42-d adult plants of the mutants were phenotyped along with the wild-type plants. Most of the mutants had enhanced seed germination (~100%) compared with the wild type (Col-0), but the differences were not statistically significant under long-day (LD) conditions (Table 3). Most mutants had longer hypocotyls than the wild type, except for one of the *ga2ox2* mutants (*gox2_3-1-1-6*) (Table 3). Three *ga2ox3* mutants displayed longer roots consistently, whereas there were large variances for root length among mutants of other individual genes (Table 3). Seed abortion was observed in the ga2ox2 mutants (see below), and it led to lower germination of *gox2_3-1-1-6* (91.52%), but germination, hypocotyl, and root length of the other two mutants were unaffected (Table 3). *ga2ox6*, *ga2ox7* and *ga2ox8* displayed an increased plant height, however, only the *ga2ox8* plants were significantly taller than the wild-type plants (Fig. 20A). Arabidopsis flowering time is presented with days under light and/or the number of rosette leaves when the first flower buds are visible. Under LD conditions, *ga2ox4* and *ga2ox8* plants flowered one-two days earlier than the wild type, and the differences were significant both when measuring the number of days and when measuring the number of rosette leaves on the main stem at flowering (S4 Fig.). One of the *ga2ox3* lines (*gox3_12-3-27*) and one of the *ga2ox6* lines (*gox6_9-6-3-7*) flowered earlier than the wild type, and it was only significant when measuring the number of leaves (S4 Fig.). Again, inconsistent results

were obtained from two independent *ga2ox2* lines: *gox2_3-1-1-6* flowered significantly earlier (1.4 d earlier on average) but with significantly more rosette leaves (1.2 more leaves on average) than the wild type; *gox2_8-2-3-16* flowered earlier only when number of leaves was considered (S4 Fig.). Flowering time of the rest of the mutants was similar to that of the wild type (S4 Fig.). Interestingly, the *ga2ox2* lines displayed a significantly increased number of siliques on the main stem and developed more high-order shoots (Fig. 20B). In addition, we observed potential defects in flower and/or silique development of the *ga2ox2* lines (see below).

Table 3. Seedling phenotypes of the *ga2ox* single knockout mutants

Genotype	Accessions	Germination (%)	Hypocotyl Length (cm)	Root Length (cm)
wild type	Col-0	96.25 ± 2.75	0.20 ± 0.01	0.68 ± 0.13
ga2ox1	*gox1_10-1-2-3*	93.73 ± 1.93	0.24 ± 0.01	0.93 ± 0.17
ga2ox2	*gox2_5-2-7*	99.16 ± 0.06	0.27 ± 0.02	1.06 ± 0.16
	gox2_3-1-1-6	99.71 ± 0.51	0.24 ± 0.00	0.65 ± 0.04
	gox2_8-2-3-16	91.52 ± 2.77	0.20 ± 0.01	0.35 ± 0.03
ga2ox3	*gox3_12-11-34*	99.74 ± 0.45	0.25 ± 0.01	0.95 ± 0.05
	gox3_10-1-8	99.42 ± 1.00	0.22 ± 0.03	0.94 ± 0.10
	gox3_12-3-27	98.67 ± 1.03	0.22 ± 0.04	0.89 ± 0.10
ga2ox4	*gox4_4-6-43*	99.06 ± 0.82	0.26 ± 0.00	0.85 ± 0.03
ga2ox6	*gox6_9-6-3-7*	99.52 ± 0.42	0.27 ± 0.02	0.99 ± 0.24
	gox6_9-6-1-3	99.10 ± 0.37	0.23 ± 0.02	0.69 ± 0.08
ga2ox7	*gox7_18-7-13*	99.75 ± 0.43	0.22 ± 0.02	0.58 ± 0.30
	gox7_18-7-2	100 ± 0	0.24 ± 0.02	0.65 ± 0.16
	gox7_9-7-9	99.61 ± 0.67	0.24 ± 0.02	0.78 ± 0.18
	gox7_7-3-22	99.08 ± 0.35	0.25 ± 0.01	0.86 ± 0.17
gox8	*gox8_12-9-9*	97.74 ± 0.83	0.27 ± 0.00	0.91 ± 0.05
	gox8_6-1-14	97.69 ± 1.93	0.22 ± 0.02	0.52 ± 0.15
	gox8_4-4-6	97.95 ± 1.57	0.26 ± 0.01	0.65 ± 0.09

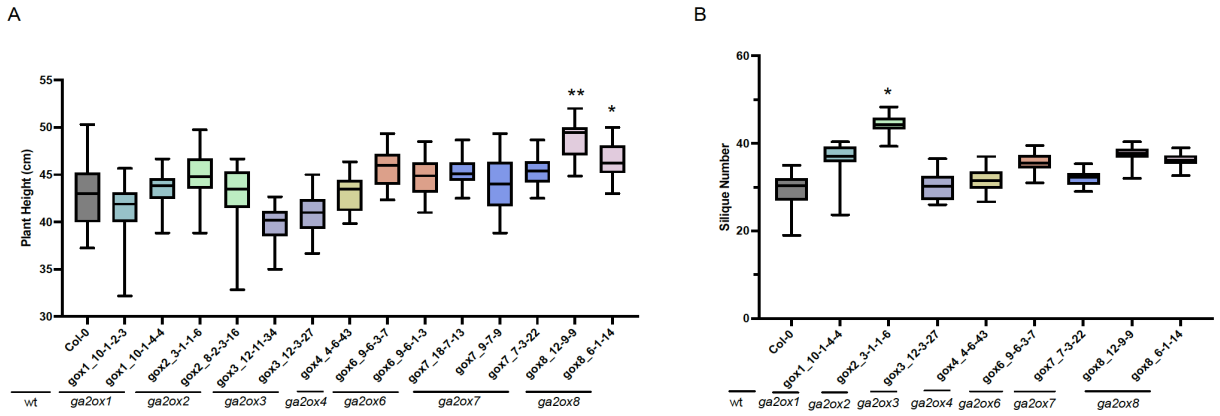

Figure 20. Adult plant phenotypes in the *ga2ox* mutants. Two box plots show the plant height (A) and silique number (B) on the main stem of each genotype including the wild type (Col-0). Plants were 42 d old grown on soil under long-day conditions. Two-tailed t-test with unequal variance was used for significant analysis. Asterisks represent statistical significance compared with the wild type: * denotes significance at $\alpha = 0.05$, ** at $\alpha = 0.01$.

Construction of transcriptional and translational GUS-fusion lines

To generate a toolkit that can be used to visualize gene expression, we generated GUS-fusion lines for each of the seven *AtGA2ox* genes. The transcriptional constructs (*pGA2ox::GUS*) contained 1-kb of the promoter region and the entire 5'-untranscribed region (5'-UTR) to the ATG translational start site fused with the GUS-encoding gene *gusA*. The transcribed regions of several GA metabolic genes are required for their proper expression (Silverstone et al. 1997; Itoh et al. 1999; Sakamoto et al. 2001). It is unknown if the intact transcribed region or just introns are essential, even though the transcribed region of *GA2ox* is conventionally included in the GUS reporter systems, either partially or entirely (Jasinski et al. 2005; Bolduc and Hake 2009; Li et al. 2019). To assess whether the transcribed region of *GA2ox* influences gene expression, we also generated translational GUS-fusion lines. The translational constructs (*pGA2ox::GA2ox-GUS*) contained the 1-kb promoter sequence and the 5'-UTR as well as the intact transcribed region without the stop codon, which comprised three exons and two introns for each

gene (Fig. 21). To avoid the chimeric GA2ox-GUS protein from affecting the stability and activity of GUS, a flexible linker sequence encoding ASGGGA was fused between the *GA2ox* gene and the *gusA* gene (Fig. 21; linker, Koo et al. 2007). The *GUS-only* construct containing the *gusA* gene only was used as a control (Fig. 21).

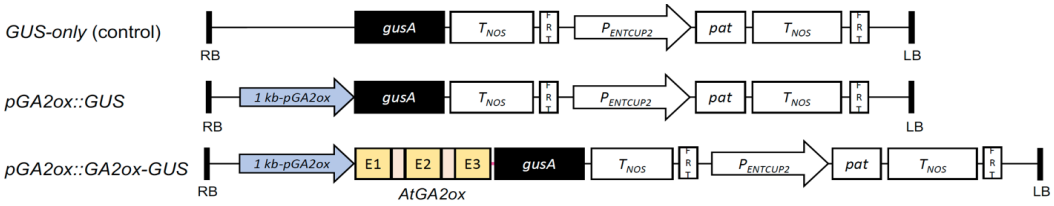

Figure 21. Illustration of GUS-fusion construct structures. Each of the *GA2ox* genes was used for one transcriptional (*pGA2ox::GUS*) construction and one translational (*pGA2ox::GA2ox-GUS*) construction. The translational constructs contain 1 kb of promoter region and the intact gene (without the stop codon), including three exons and two introns, fused to the amino terminus of the GUS reporter gene (*gusA*). The transcriptional constructs contain 1 kb of promoter sequence upstream of the translational start site fused to GUS. E1-3 represent three exons. Refer to Coutu et al. 2007 for details of vector pORE-O3. The short red line between *GA2ox* and *gusA* represents an 18-bp linker that encodes amino acids ASGGGA.

At least eight independent lines for each *GA2ox-GUS* fusion and 20 independent *GUS-only* lines were obtained (data not shown). As a first step, we checked if there was any GUS activity in the *GUS-only* lines that did not contain a promoter and found that no GUS activity was detected in most of the lines. Only two *GUS-only* lines showed GUS activity, which might reflect the influence of the neighboring sequences of the insert, given the randomness of T-DNA-based insertion in the genome. We then assessed the consensus expression of each transcriptional and translational *GA2ox-GUS* fusion lines by comparing GUS activity in 16-d seedlings and in the inflorescences of 35-d plants. At least three independent lines with consistent expression were obtained for most constructs, except for *pGA2ox7::GUS* and *pGA2ox7::GA2ox7-GUS* (Fig. 22).

GUS activity in individual transcriptional and translational GUS-fusion lines was compared, using as reference the compiled gene expression data obtained from the Arabidopsis eFP Browser based on microarray and transcriptome data (herein, called the database data) (S6 file) (Klepikova et al. 2016; Schmid et al. 2005). Where GUS activity was detected, the expression patterns were generally consistent with the database data (Fig. 22). Differences in GUS activity were observed between the translational and transcriptional lines of most genes (except for *GA2ox3*, see below). For some genes such as *GA2ox2*, *GA2ox7* and *GA2ox8*, the translational lines had more consistent expression patterns with the database and stronger GUS activity than the transcriptional lines, indicating transcriptional and/or post-transcriptional control involving the transcribed region (Fig. 22). However, for others such as *GA2ox1*, *GA2ox4* and *GA2ox6*, it was the opposite, suggesting that some unknown regulation was present in the translational lines (Fig. 22) (see below). No GUS activity was observed in seedlings or inflorescences of the *GA2ox1* translational lines (*pGA2ox1::GA2ox1-GUS*); in contrast, only faint GUS stain was found in the inflorescences of the transcriptional lines, more specifically, in pedicel, stem, stamen and pistil, with the highest level in pedicel (Fig. 22). A relatively strong GUS activity in the *GA2ox2* translational and transcriptional lines was found in the shoot apex and young true leaves of seedlings as well as in flower organs and young siliques of adult plants, with the highest level of activity in sepal and young siliques (Fig. 22). The *GA2ox2* transcriptional lines showed reduced GUS activity in most structures examined here, most prominently in the shoot apex, young true leaf, carpel, pedicel, and stem (Fig. 22). The *GA2ox3* transcriptional and translational lines had similar and specific GUS activity patterns with the strongest activity in the stamen of unopened flower buds and embryos

(Fig. 22). *GA2ox4* was also active in the stamen (but in a specific substructure of stamen) and embryos (Fig. 22). *GA2ox6* was highly expressed in root, hypocotyl, stem, and stamen (Fig. 22). In addition, *GA2ox4* and *GA2ox6* had overlapping expression in the abscission zone of petals and sepals and/or nectarium (Fig. 22). *GA2ox4* and *GA2ox6* sequences are more similar to each other than other genes. It is not clear if these two genes were originally duplicated from one another, but their specific expression in this structure may suggest an overlapping role. Various levels of GUS activity were observed among the *GA2ox7* lines, but the highest level was found in the ovule (Fig. 22). *GA2ox8* was highly active in the root, petals of young flower buds, pistils of open flowers, young siliques, and the stem (Fig. 22). Finally, our data supports that *GA2ox2* and *GA2ox8* may be expressed in guard cells as reported by Li et al. (2019), represented with the scattered patterns of GUS activity in young leaves and the stem (Fig. 22).

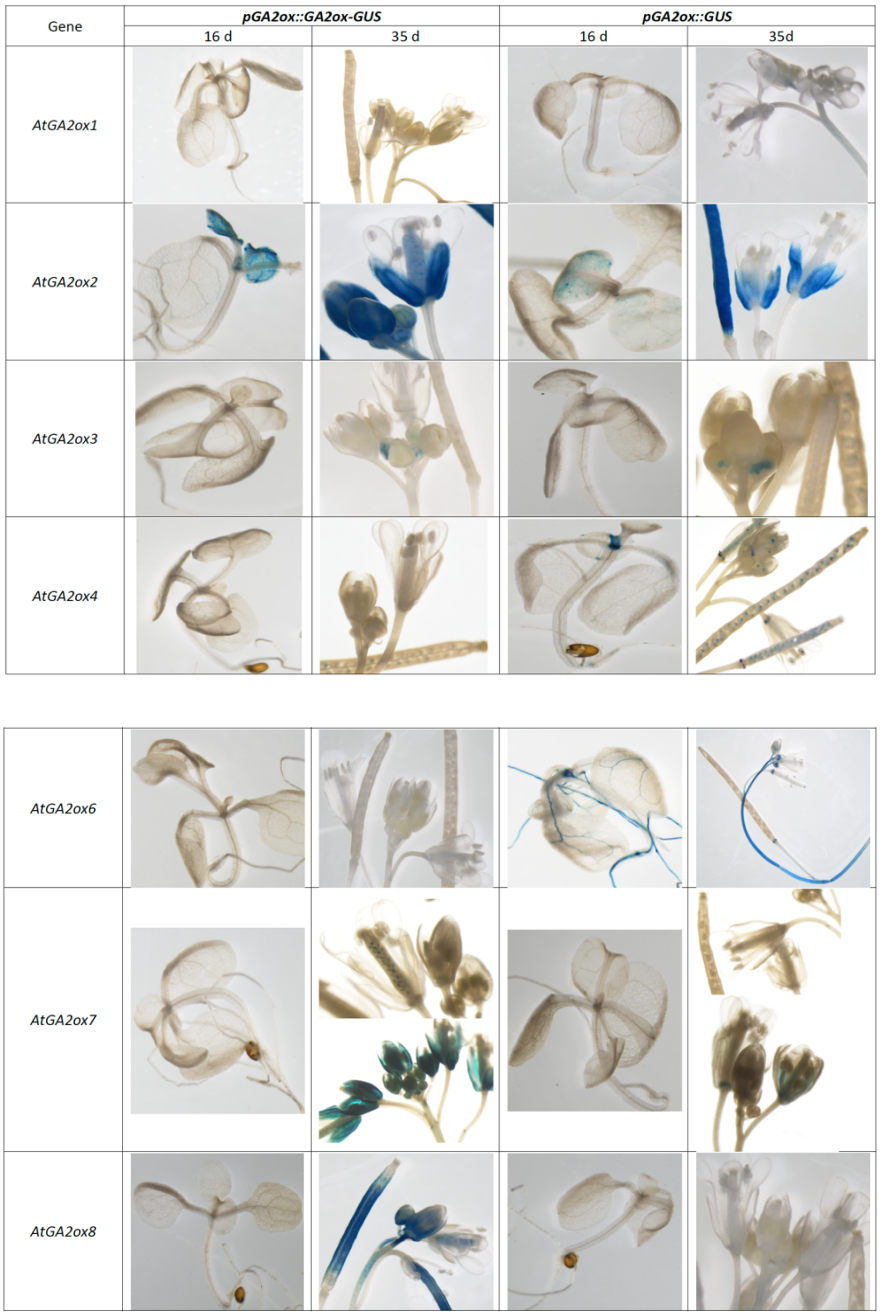

Figure 22. GUS activity consensus in *GA2ox-GUS* transcriptional and translational lines. Where no consensus GUS activity was represented by at least three independent lines, two representative pictures were given.

GA2ox2 regulates multiple developmental processes

GA2ox2 is one of the most highly expressed *GA2ox* genes in Arabidopsis, and it is expressed ubiquitously throughout the plant and throughout the life cycle (S6 file; Schmid et al. 2005; Rieu et al. 2008; Klepikova et al. 2016). As discussed above, we found that loss of function of *GA2ox2* led to several GA-associated phenotypes (e.g., altered flowering behavior, defects in flower and/or seed development). We proposed that *GA2ox2* plays more widely roles in plant growth and development than what has been acknowledged (root meristem cell number, seed germination, cytokinin and GA crosstalk in the shoot apical meristem; see Chapter 1). To study its function, we re-phenotyped two *ga2ox2* mutants along with the wild type. Consistent with the results above, *gox2_8-2-3-1*6 flowered around the same time as the wild type, whereas *gox2_3-1-1-6* flowered significantly earlier (2.4 d; Fig. 23). Despite the inconsistent flowering time, defects in flower development were observed in both *ga2ox2* mutants. Specifically, a significant portion of pistils failed to push out of the petals and/or sepals and thus were bent. Petals and sepals were attached to the pistil during most of the reproductive stage. As a result, infertility and thus seed abortion was widely found in the *ga2ox2* mutants (Fig. 24). This resembled a phenotype that was previously observed in a *ga2ox quintuple* mutant containing mutations in *GA2ox1*, *-2*, *-3* , *-4* and *-6* (Rieu et al. 2008). It was proposed that unbalanced GA levels in pistil and sepals or petals resulting from loss of *GA2ox* function contributed to their uncoordinated growth. Our results showed that knock-out of a single *GA2ox* gene, *GA2ox2*, can cause the phenotype that was observed in the *ga2ox quintuple* mutant, suggesting *GA2ox2* is a major player in the coordinated growth of flower organs. As discussed above, *GA2ox2* was predominantly expressed in sepals. Therefore,

we suspect that *GA2ox2* affects flower development mainly through regulating sepal development.

To have supporting expression data, we also grew several *GA2ox2-GUS* fusion lines with consensus GUS activity along with the mutants and the wild type. Interestingly, whatever phenotypes we observed in the *ga2ox2* mutants, we saw the opposite in the *GA2ox2* translational lines: semi-dwarfism, small dark green leaves, reduced rosette radius, significantly delayed flowering, reduced number of flowers, siliques, and high-order shoots (Figs. 23, 25, 26 and S5 Fig.). These traits resembled characteristic GA-deficient phenotypes but were not seen in the transcriptional lines, which did not show any apparent phenotypic differences than the wild type (Figs. 23 - 26). The translational lines were homozygous for the inserted construct, and thus contained two additional intact copies of the *GA2ox2* gene. As a linker was infused between *GA2ox2* and *GUS*, our results suggest that GUS activity was not affected by *GA2ox2*, and vice versa. We suspected that two additional copies encode a functional GA2ox2 enzyme, which resulted in GA-deficient phenotypes in the *pGA2ox2::GA2ox2-GUS* lines, and that the *pGA2ox2::GA2ox2-GUS* lines were actually *GA2ox2* overexpression lines with the additional copies of *GA2ox2* driven by its native promoter and fused with a reporter gene *GUS*. Indeed, in contrast to the early-flowering phenotype of *ga2ox2* (specifically *gox2_3-1-1-6*), the two independent *GA2ox2-GUS* translational lines showed significantly delayed flowering, dwarfism, small dark leaves, reduced number of flowers, siliques, and high-order shoots (Figs. 23, 25, 26 and S5 Fig.), the opposite to the phenotypes observed in the *ga2ox2* phenotypes. It is worth noting that even though fruit set was reduced in the *GA2ox2-GUS* translational lines, there was no obvious evidence indicating seed abortion

(Fig. 24), which indicates that *GA2ox2* influences seed development indirectly by regulating sepal development. Collectively, the results supported that *GA2ox2* regulates numerous growth and developmental processes, including leaf expansion, flowering, flower, and seed development, and shoot architecture.

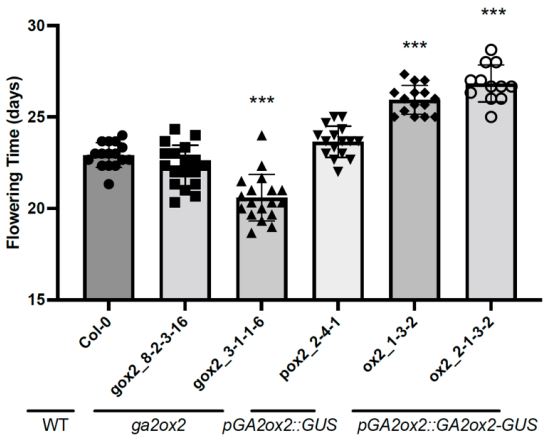

Figure 23. Flowering time of *GA2ox2*-associated lines and the wild-type plants represented with days at flowering. As above, two-tailed t-test with unequal variance was used for significant analysis. Asterisks represent statistical significance compared with the wild type: * denotes significance at α = 0.05, ** at α = 0.01. Error bars were standard deviations of plants in three biological replicates.

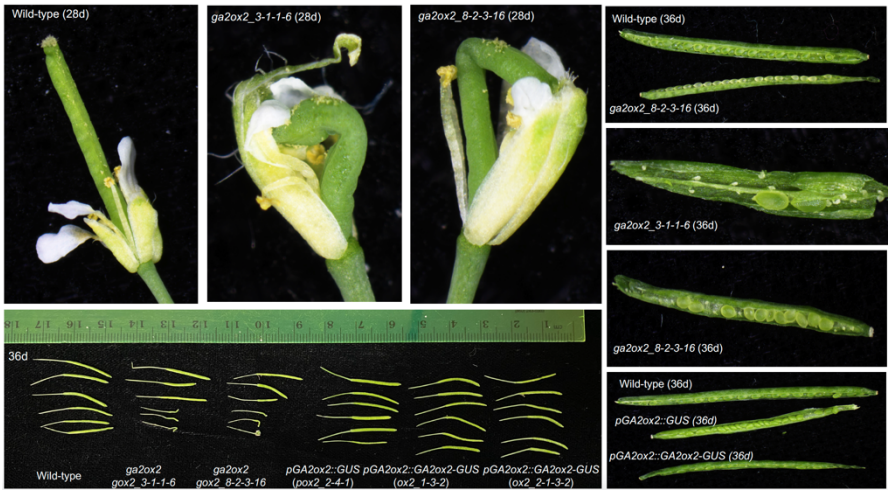

Figure 24. Silique and seed development of the wild type and *GA2ox2*-associated lines at 28 d (top left) and 36 d (the rest).

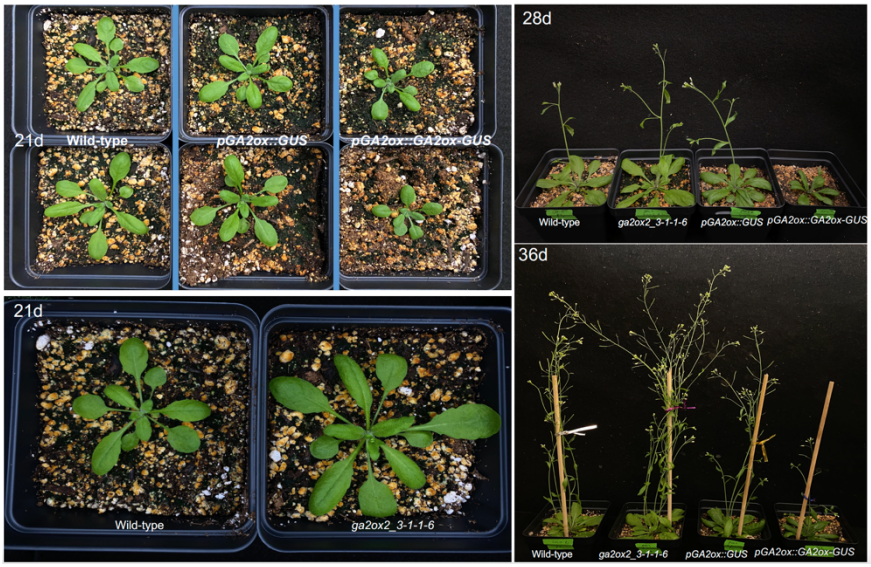

Figure 25. Whole-plant development of the wild type and *GA2ox2*-associated lines at 21 d (left), 28d (top right) and 36 d (bottom right).

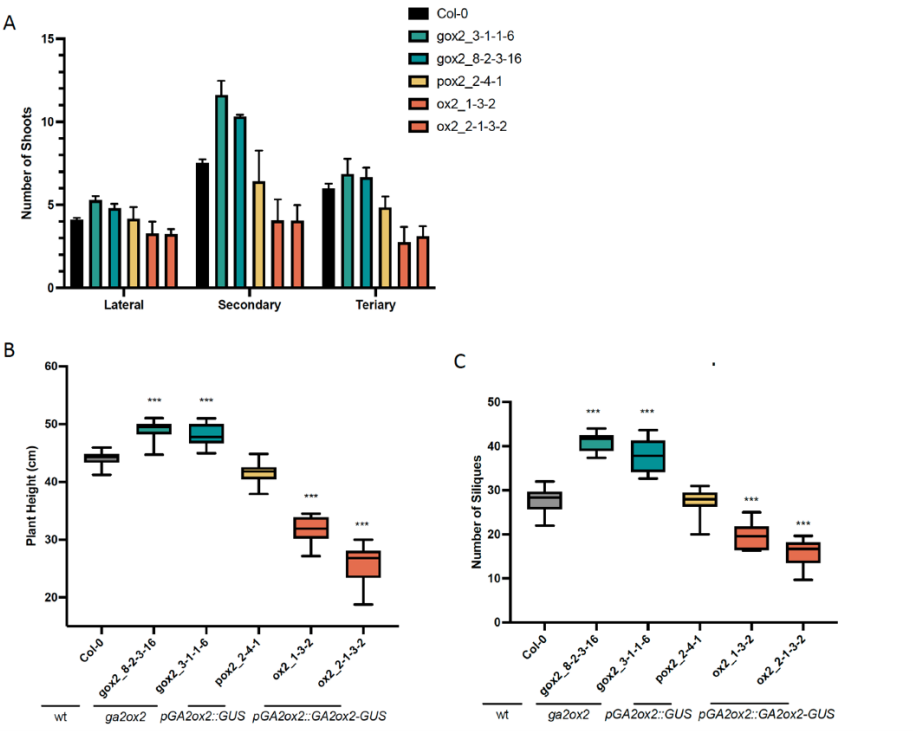

Figure 26. Altered expression of *GA2ox2* affected shoot structure, plant height, and fruit set. A. Number of lateral, secondary, and tertiary shoots; B. Plant height; C. Number of siliques on the main stem. Asterisks represent statistical significance compared with the wild type: *** denotes significance at α = 0.001. Error bars were standard deviations of plants in three biological replicates.

Discussion

CRISPR single knockout mutants and their phenotypes

Single T-DNA insertion mutants of *GA2ox1*, *-2*, *-3*, *-4*, and *-6* showed no apparent phenotypes due to function redundancy, and thus a quintuple mutant containing mutations in was developed by Rieu et al. (2008) for *GA2ox* function studies. Even though it is not clear if the presence of the T-DNA inserts and its intragenic insertion locations influence accurate interpretation of gene function, to evaluate specific function of each gene and to reduce noise as much as we can, we developed CRISPR/Cas9-induced transgene-free biallelic homozygous single knockout lines for each of seven Arabidopsis *GA2ox* genes (*GA2ox*1-*GA2ox8*) (Fig. 19). These transgene-free single loss-of-function mutants can be readily used for future *GA2ox*- and/or GA-related studies, and higher-order transgene-free knockout mutants can be readily developed through crossing.

Three genes, *GA2ox2*, *-4* and *-6*, are expressed in the shoot apex (Wang et al. 2004; Jasinski et al. 2005; Eriksson et al. 2006). By comparing phenotypes of a combination of mutants (single, double, triple and quintuple), Rieu et al (2008) found that the early flowering phenotype of the quintuple mutant was mainly credited to knockout of *GA2ox4*, and that the other two genes may play a minor role. Indeed, we found *ga2ox4* mutants flowered significantly earlier than the wild type in LDs (S4 Fig.). We did not observe the early flowering phenotype in the *ga2ox*6 mutants (S4 Fig.). However, two *ga2ox2* mutants showed inconsistent flowering behavior: one mutant (*gox2_3-1-1-6*) flowered early and developed more rosette leaves at flowering, while another (*gox2_8-2-3-16*) flowered normally with less rosette leaves (S4 Fig. and Fig. 23). When comparing other phenotypes, we found that *gox2_3-1-1-6* always had more prominent excess-GA

phenotypes than the other line, including rosette radius, leaf size and silique number (Fig. 25). The *ga2ox2_8-2-3-16* seeds used in the phenotyping experiments looked smaller and some of them were unfertilized. Therefore, it may be due to maternal effects and should be tested again with healthy next-generation seeds.

It has been reported that overexpression of *GA2ox8* led to dwarfism while loss of *GA2ox8* resulted in elongated hypocotyl, slightly late flowering in LDs and much later in short days (SDs), indicating *GA2ox8* is responsible for hypocotyl and/or stem elongation and regulating flowering behavior (Schomburg et al. 2003). Our initial mutant phenotyping showed that *ga2ox8* mutants displayed enhanced plant height and early flowering in LDs (Fig. 20 and S4 Fig.), which agrees with others' findings (Schomburg et al. 2003). However, it is unknown if these phenotypes reflect a direct role of *GA2ox8* in these developmental processes through regulation at the transcriptional level or an indirect role of *GA2ox8* through reducing internal GA levels.

Otherwise, we did not observe apparent phenotypes in other mutants. It was found that some *ga2ox* mutants including the *ga2ox quintuple* mutant displayed more sharp GA-associated phenotypes under GA-limiting conditions such in SDs, under GA inhibitor treatments and even under certain abiotic stresses (e.g. cold, drought and salt) (Schomburg et al. 2003; Rieu et al. 2008; Lange et al. 2020). These agitating conditions should be considered and included to enable future mutant phenotyping. In addition, tissue-specific gene expression can guide phenotyping work (Fig. 22). According to GUS activity observed in the *GA2ox4-GUS* fusion lines, *GA2ox4* was highly specifically expressed in a certain substructure of stamen. It would be of interest to examine *GA2ox4*'s function in stamen development. Even though *GA2ox6* is another highly

expressed gene in addition to *GA2ox2*, the phenotypes conferred by loss of this gene might have been subtle or missed in the present study. For example, it has been recently reported that *GA2ox6* is expressed in mature, secretory nectaries and that loss of *GA2ox6* resulted in reduced nectar production (Wiesen et al. 2016). Another notable example of tissue-specific expression is *GA2ox8* in guard cells. A recent preprint reported that a tomato *GA2ox* gene, *SlGA2ox7*, was induced in guard cells by water deficit and that the tomato *ga2ox7* mutant was hyposensitive to soil dehydration, as loss of this gene delayed stomatal closure (Shohat et al. 2021). Interestingly, *SlGA2ox7* is a homolog of A*tGA2ox8* (Chen et al. 2016). Despite the guard cell-specific expression, whether *GA2ox8* is involved in regulating stomatal closure in response to drought remains uncharted in Arabidopsis.

GUS-fusion lines

To assess if the transcribed region contains any regulatory elements that are essential for transcriptional or post-transcriptional gene regulation, we developed both transcriptional and translational GUS fusion lines for each of seven *GA2ox* genes (Fig. 21). We found that transcriptional lines of *GA2ox2*, *-7* and *-8* displayed reduced GUS activity compared with the transcriptional lines, whereas it was the opposite for *GA2ox1*, *-4* and *-6*, indicating presence of essential *cis*-acting elements in the transcribed region (Fig. 22). It has been reported that the transcribed region is required for proper expression of several GA metabolic genes, and transcriptional GUS fusions of these genes failed to show consistent GUS activity (Silverstone et al. 1997; Itoh et al. 1999).

The involvement of the transcribed region is not only limited to GA metabolic genes. The transcriptional GUS fusion of an auxin efflux carrier gene, *EIR1*, conferred GUA

activity in the entire root, whereas its full-length translational fusion had GUS activity specifically in the root meristem, which is more consistent with the RT-PCR data (Sieberer et al. 2000). Thus, absence of its transcribed region failed to confer tissue-specific expression. Similarly, the intragenic sequences are also found to be required for tissue-specific expression of a MADS box gene, *AGAMOUS* (Sieburth and Meyerowitz 1997). For *GA2ox1*, *-4* and *-6*, lacking the transcribed region conferred a higher GUS activity, and it may be partially attributed to GA feedforward regulation caused by overexpression of these genes. *GA2ox3* expression was not affected by the inclusion or lack of the transcribed region (Fig. 22).

A recent study documented expression of the same seven Arabidopsis *GA2ox* genes using translational GUS fusion lines that contained the entire 5' -upstream sequence from the start codon of *GA2ox* up to the upstream neighboring gene upstream along with the entire transcribed region (Li et al. 2019). In contrast, our translational constructs included the truncated promoter sequence (Fig. 21). It is unknown if the entire intergenic region is required or if the 1-kb promoter is sufficient to drive proper gene expression. But when comparing GUS activity in two different groups of transgenic, we observed consistent GUS activity for *GA2ox2*, *-3* and *-8* in the translational GUS fusion lines (Fig. 22). Reduced or no GUS activity was found in the translational lines for the rest of genes (*GA2ox1*, *-4* and *-6*) (Fig. 22), which is inconsistent with Li et al. (2019). Even though similar GUS activity was observed in the transcriptional lines of these genes, GUS activity was not as strong as the lines generated by Li et al, for example, reduced activity of *GA2ox*1 in seedlings and inflorescence, and *GA2ox7* in seedlings (Fig. 22). It suggests that the 1-kb promoter may not have been sufficient for their expression.

GA2ox2 and its functions

When growing the *GA2ox2-GUS* fusion plants along with *ga2ox2* and the wild-type plants, we observed GA-deficient phenotypes in the GUS fusion lines. It was unexpected but reasonable, given the facts that *GA2ox2* is widely expressed and that GA2ox2 mainly uses as substrate the predominant bioactive GA form in Arabidopsis, GA_4 (Thomas et al. 1999). In addition, expression of *GA2ox2* is under GA feedforward regulation (Rieu et al. 2008). Even though a new GA homeostasis might have been achieved through regulation of *GA2ox* and other components, the apparent GA-deficient phenotypes alarmed that the plants had generally low bioactive GA levels. Nevertheless, we did not see reduced GUS activity in this specific background (Fig. 22). It is not impossible that the GA-responsive *cis*-acting elements reside upstream of the 1-kb promoter included the translational GUS fusion construct. It is not clear if the *GA2ox2* translational plants that Li et al. (2019) developed had phenotypes as ours, as there was no phenotypic documentation. It is also not immediately clear to us if the translational lines for the rest of genes have GA-associated phenotypes. The *GA2ox2-*, *GA2ox3-* and *GA2ox8-GUS* fusion lines (where truncated promoter was sufficient for their expression) containing two additional functional copies of each gene and the single knockout mutants lacking any functional single *GA2ox* may provide unique opportunities for future studies on their specific function.

Based on the levels of GUS activity in the structures examined here, *GA2ox2* was one of the most highly and widely expressed *GA2ox*, which was consistent with others' studies (Wang et al. 2004; Rieu et al. 2008). Knockout of *GA2ox2* led to several apparent excess-GA phenotypes, indicating that *GA2ox* is involved in multiple developmental processes including leaf expansion, flower development, fruit set, stem elongation and

shoot establishment (Figs. 23-26). Meanwhile, expressing two additional functional copies of *GA2ox2* resulted in GA-deficient phenotypes that were opposite from the knockout mutants, which further strengthened *GA2ox2*'s roles in these processes (Figs. 23, 25 and 26). Future directions include studying if the translational constructs could recover the gene expression in their corresponding knock-out mutant lines and if loss of function and native overexpression can confer any other apparent or subtle defects in growth and development under agitating conditions such as SDs, cold, drought and salinity. In addition, future studies should enable a better understanding of how *GA2ox2* is involved and regulated in these processes.

Methods

Plant material and growth conditions

The wild-type backgrounds of all plants used in this study were Col-0. Unless stated otherwise, seeds were stratified at 4 °C in dark for 4 days before being sown on soil (SunGro Professional Growing Mix) in trays, comprising 10 large square pots (size: 10 cm * 10 cm * 10 cm) with four seedlings in each pot for harvesting seeds or 18 small square pots (size: 8.2 cm * 8.2 cm * 8.4 cm) with one seedling each for experiments under long-day conditions, specifically 16h light at 100 mmol m^{-2} s^{-1} from Philips white fluorescent lights (32-watt, model F32T8/TL741), 25 °C, 70 % relative humidity/8 h dark, 18 °C, 70% relative humidity). Plants were watered regularly and fertilized with a standard plant nutrient solution once per week.

For seedlings grown on medium, seeds were surface sterilized with 70% ethanol for 2 min followed by 50% bleach for 5 min and by at least four washes with sterile ddH_2O. Seeds were stratified at 4°C in dark for 4 days before being sown on half-strength

Murashige and Skoog (MS) agar square petri dishes and then placed vertically in a controlled environment growth room (16-h light 200 mmol m^{-2} s^{-1}/8-h dark cycle at 22°C/20°C).

Genomic DNA extraction

A protocol for genomic DNA extraction was modified from a previous method (Murray and Thompson 1980). Briefly, two youngest leaves of vegetative plants were harvested into 1.7 mL microcentrifuge tubes containing 300 mL of 2X CTAB buffer (prewarmed at 65°C). Tissues were ground at room temperature with plastic pestles driven by a drill (DeWALT) before being incubated at 65°C for 15 min. Genomic DNA was then extracted in 300 uL of chloroform and precipitated with 300 uL of 2-propanol. The resulting pellet was washed with 500 uL of 70% ethanol and dissolved in 50 uL of TE buffer.

Construction of GUS expression vectors

SLiCE (Seamless Ligation Cloning Extract) cloning (Zhang et al. 2012, 2014) was used to insert the GUS coding sequence into the pORE-O3 vector. The GUS coding sequence was amplified from the pBI101 vector using Phusion DNA Polymerase (New England Biolabs), and oligonucleotide primers with partial homology with termini of the linearized vector pORE-O3 (S6 Table). The pORE-O3 plasmid containing a selectable marker, *phosphinothricin acetyltransferase* (*pat*) gene driven by a tobacco cryptic constitutive promoter ($P_{ENTCUP2}$) (Coutu et al. 2007), was obtained from donation. PPY SLiCE extract and 10x SLiCE buffer were prepared according to Zhang et al. 2014. The reaction system consisted of 100 ng vector DNA, 200 ng insert DNA, 1 uL 10x SLiCE buffer, 1 uL SLiCE extract, and 6 uL ddH_2O. The reaction mixture was incubated at 37°C

for 15 min, and then 1 uL was used for transformation of *E. coli* (*DH10B*, Invitrogen). Transformants were screened using PCR with M13 forward and reverse primers (S6 Table). Plasmid DNA, pORE-O3-GUS, was purified and subjected to double restriction digestion with PstI-HF and EcoRI-HF (NEB) or Sanger sequencing to confirm the insertion of GUS. Primers used for Sanger sequencing were M13 forward and reverse primers, and GUS-primers 1-4 (S6 Table). *GA2ox* genomic sequences were amplified from genomic DNA extracted from wild-type leaves of accession Col-0, using AtGA2ox and pAtGA2ox primers (S6 Table). Transcriptional (promoter-only, *pox* lines) constructs were engineered such that promoters were inserted upstream and immediately adjacent to GUS in a SLiCE reaction. To engineer the translational constructs (*ox* lines), a pair of primers (pORE-O3-linker-GUS forward and reverse, S6 Table) were used to linearize the pORE-O3-GUS vector and add a linker sequence (GCT AGC GGA GGA GGT GCC). The linearized modified vector was then ligated to *GA2ox* genomic sequences in a SLiCE reaction. The recombinant constructs were subjected to diagnostic digestion with restriction enzymes from the New England Biolabs (BglII for *pOX1*, *pOX8*, *OX1*, *OX2, OX4*, *OX6* and *OX8*; BbSI for *pOX2*, EcoRI-HF for *pOX3*, BamHI-HF and BglII for *pOX4* and *pOX7*, SacI-HF for *pOX6*; HindIII for *OX3*; BamHI-HF for *OX8*) and Sanger sequencing with a list of primers: specifically, M13 forward and GUS-primer 5 were used for sequencing all transcriptional constructs; in addition to M13 forward and GUS-primer 5, 2-5 more primers were designed to cover the body of each gene for refined sequencing of translational constructs (S6 Table). Recombinant plasmids were then introduced into *Agrobacterium tumefaciens* (strain *GV3101*). Agrobacterium cells with recombinant

plasmids were selected and cultured in LB medium containing 50 ug/mL kanamycin, and then used to inoculate Arabidopsis plants by floral dipping (Clough and Bent 1998).

Development of homozygous GUS-fusion lines

At least 12 healthy adult plants with most flower buds unopened were transformed using the floral dip method (Clough and Bent 1998). T1 seeds from individual T0 plants were harvested separately and grown on soil for 7-10 days before being sprayed for three consecutive days and selected with 10 mg/mL glufosinate (BASTA) for transformants containing the kanamycin-resistance gene. Approximately 100 of T2 seeds were used to determine the zygosity of the individual T1 plants. T3 seeds derived from the T1 lines that may be heterozygous for the construct insertion were kept and plants were selected again to determine homozygous T2 lines. Where homozygous T2 lines were not identified at the T3 generation, T4 seeds were harvested, and plants selected until a homozygous line derived from an individual T1 line was identified. Sizes and sequences of the insertions in homozygous transgenic lines were confirmed with PCR with M13 forward and reverse primers plus gel electrophoresis and Sanger Sequencing.

GUS staining

Plant tissues were harvested either in glass containers (e.g., whole plants) or microcentrifuge tubes (e.g., inflorescences and young seedlings) containing 90% acetone on ice. When all harvested, samples were let sit at room temperature for 20 min before acetone was removed. Samples were rinsed with the staining buffer that does not contain X-Gluc (0.2% Triton, 50 mM pH 7.2 $NaHPO_4$ buffer containing 1M Na_2HPO_4 and 1M NaH_2PO_4, 2mM potassium ferrocyanide and 2mM potassium ferricyanide) before the staining buffer containing 1 - 2 mM X-Gluc was added. Samples were vacuumed on ice

for 20 min and incubated at 37°C overnight. After incubation, staining buffer was removed and tissues were incubated in 20%, 30% and 50% ethanol, FAA buffer (10 mL FAA buffer contains 5 mL of ethanol, 1.37 mL of 36.5% formaldehyde, 1 mL of acetic acid and 2.63 mL ddH_2O), and 70% ethanol, respectively, each for 30 min. Tissues were cleared in several 5-10 min incubations in 95% warm ethanol (prewarmed in 65°C water bath) before being observed in ethanol under a Stereo microscope (Nikon SMZ 800).

Construction of CRISPR/Cas9 gene editing vectors

A binary plasmid vector, pHEE401E (Addgene #71287), was used to express a Cas9 gene which was driven by an egg cell-specific promoter (*EC1.1p*) and enhancer (*EC1.2en*) and two guide RNAs (Fig. 18). Vector pHEE401Elt has two BsaI cloning sites and a scaffold RNA (tracrRNA) sequence, which allowed the use of Golden Gate Cloning and expression of gRNAs and Cas9 in the same vector with a 1:1 molecular ratio. This vector has bacterial resistance to kanamycin and a selectable marker hygromycin in plants. The size of this plasmid was around 17 kb. pHEE401E has a U6-26 promoter and a U6-26 terminator to regulate the expression of one gRNA. To express another gRNA, one copy of U6-1 promoter and terminator was amplified from another vector (pYLsgRNA-AtU6-1) and inserted into this vector.

Sequence-specific CRISPR RNAs (crRNAs) were designed using the online tool CHOP-CHOP (Labun et al. 2019, http://chopchop.cbu.uib.no/). Specifically, two 20 bp-long sequences adjacent to a PAM (Proto-spacer Adjacent Motif) sequence (5'-NGG-3') and with a predicted high cutting efficiency and low off-target potential were chosen. The protocol for the assembly of the two crRNAs was modified from Wang et al. (2015). Specifically, four primers were designed to amplify scRNA, AtU6-1 promoter and

terminator from pYLsgRNA-AtU6-1 (Addgene plasmid # 66202) and to add one crRNA and a BsaI cloning site to both ends of the PCR product. The amplification reaction contained a Phusion PCR reaction with F0 and R0 primers 20x less than BsF and BsR primers (S6 Table). A 15 uL Golden Gate reaction was set up as follows: 2 uL purified PCR fragments (~100 ng/uL), 2uL plasmid (~100 ng/uL), 1.5 uL 10x T4 DNA ligase buffer (NEB), 1 uL T4 DNA ligase of high concentration (2,000,000 units/mL, NEB), 1 uL BsaI-HFv2 (NEB), and 6 uL ddH_2O. The reaction conditions were 37°C for 1 h, 50°C for 5 min, and then 80°C for 10 min. *E. coli* competent cells (DH5a, ThermoFisher Scientific MAX Efficiency™) were transformed with 5 uL of Golden Gate reaction mix, and positive clones were selected on LB agar plates with 50 ug/mL kanamycin. Positive clones containing the editing system were confirmed using colony PCR and Sanger sequencing with primers pHEE401seqF and pHEE401seqR (S6 Table). Plasmid pHEE401E was a gift from Qi-Jun Chen (Addgene plasmid # 71287). Confirmed plasmids were delivered into *Agrobacterium tumefaciens* competent cells (strain *GV3101*).

Development of Cas9-free and biallelic homozygous single knockout mutants

Plant transformation procedures were as described above. T1 seeds were harvested separately from individual floral dipped T0 plants and selected for successful insertion of the gene editing cascade on ½ MS agar medium containing 25 ug/mL hygromycin. Resistant seedlings were then transferred on soil, and T2 seeds were harvested. Cas9 was detected in T2 plants with PCR using a pair of primers (Cas9_F and Cas9_R) (S6 Table). Cas9-free T2 plants were kept and the region spanning both gRNA1 and gRNA2 target sites were amplified for each line and subjected to Sanger sequencing for effective mutations and zygosity. For those whose amplicon was too long (i.e. *ga2ox3*,

-4, *-6* and *-7*), the gRNA2 target sites were Sanger-sequenced with their corresponding reverse primers used for PCR. T3 seeds were harvested from the confirmed Cas9-free T2 plants that are biallelic homozygous for mutation (herein, *gox* lines).

Mutation detection

For convenient mutation detection in later experiments, markers were designed for the representative single knockout mutants in such a way that mutation was either detected by PCR genotyping or diagnostic digestion restriction enzymes (New England Biolabs) using primers listed in S6 Table. Specifically, for *gox1_10-1-2-3*, ga2ox1_primer 2 and ga2ox1_primer 3 were used to amplify the edited region, and the PCR product was digested with XmnI; for *gox2_8-2-3-16* and *gox2_3-1-1-6*, ga2ox2_primer 2 and ga2ox_primer 4, and Hpy99I were used for amplification and digestion; for *gox3_10-1-8*, it was ga2ox3_primer 2, ga2ox3_primer 3 and enzyme AluI; for *gox4_4-6-43*, gox4_marker_forward and ga2ox4_primer 5 were used for PCR detection; for *gox6_9-6-3-7*, ga2ox6_primer 2, ga2ox6_primer 6 and enzyme CviKI-1 were used; mutations in *gox7_7-3-9* and *gox8_12-9-9* were detected with PCR genotyping using primer sets, gox7_marker_forward/-reverse and gox8_marker_forward/-reverse, respectively.

Phenotyping

Both seedling and adult plant phenotypic analyses comprised three biological replicates. Germination rate was assessed using 100 seeds per genotype for each replicate grown on ½ MS medium in petri dishes. Seeds with visible emerged radicles was counted for germination at 4 days after sown. At 7 d, at least 17 seedlings per genotype per replicate were imaged with a camera at a fixed height and angle, hypocotyl and root length were analyzed in ImageJ (version 1.53). At least 15 adult plants per

replicate of each genotype were used for flowering time, rosette number, silique number and plant height phenotyping. The flowering time of adult plants was represented with days when the first flower bud was visible at the apex and the number of rosette leaves. The silique number on the main stem and plant height were scored at 5 weeks. For *ga2ox2* and *GA2ox2-GUS* fusion lines, adult plants of each replicate of six genotypes were phenotyped on the same day between 35-37 d (e.g., replicate-1 plants on 35 d, replicates-2 on 36 d, and replicate-3 on 37 d). Specifically, the number of primary, secondary, and tertiary shoots, plant height, and rosette radius were measured. Rosette radius was represented with the length of the longest rosette leaf measured with a ruler.

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CHAPTER FIVE

Summary

Gibberellins (GAs) are small molecules that do big things in plants – they regulate numerous plant growth and developmental processes by triggering changes in transcriptional profiles, protein, and enzymatic activities. GA's functions are generally conserved in higher plants, with a notable exception. GA promotes flowering in annual plants, whereas it has a general negative effect on flowering throughout many perennial woody plants such as apple (Blázquez et al. 1998; Southwick et al. 1995; Bukovac and Yuda 1991; Randoux et al. 2012; Goldberg-Moeller et al. 2013; Zhang et al. 2019) (Fig. 27). Given that GA's negative role in flowering is also believed to contribute to one of the major challenges in apple production, alternate bearing, it is important to understand the underlying genetic and molecular mechanisms, which was one of the goals of my dissertation (Chapter 2).

A recent study proposed that GA promotes phase change (from vegetative to reproductive stage) through direct regulation of *LFY* but inhibits later flower formation through indirect regulation of *LFY* in Arabidopsis (Yamaguchi et al. 2014). This model could help explain GA's repressive role in flowering in perennial woody plants, as floral induction in apple happens on branches and shoots that are already in the reproductive stage. However, this model depends on the regulation of LFY by GA either directly or indirectly. Our results indicate that two apple *LFY* homologs (*MdAFL1* and *MdAFL2*) were not responsive to GA in the shoot apex during floral induction, challenging this explanation for GA's negative role in apple flowering (Chapter 2). We and others showed that unlike their Arabidopsis ortholog, two apple *FT* genes are not preferably expressed in leaves (Kotoda et al. 2010; Chapter 2). Instead, *MdFT1* is predominantly expressed in the shoot apex, and *MdFT2* in fruit-associated structures, indicating diverged expression in apple.

Phylogenetic analysis revealed that *FT*-like genes in the Rosaceae family originated from two sources, and that the apple and peach genomes contain only one of the two clade groups of *FT* with the more ancient cluster missing (Mimida et al. 2012). Therefore, it is tempting to propose that apple *FT* genes might have evolved with diverged function, and spatial and temporal expression. Despite being predominantly expressed in the shoot apex, *MdFT1* was not inducible by exogenous GA in the Gala or Honeycrisp shoot apex during floral induction, suggesting that it does not function downstream of the GA signaling pathway.

Instead, we found that two copies of a floral repressor gene, *MdTFL1-1* and *MdTFL1-2*, were significantly upregulated by GA in the shoot apex of 'Gala' and 'Honeycrisp' (Chapter 2). The facts that the *MdTFL1* genes were predominantly expressed in the shoot apex and that their expression was responsive to both exogenous GA and the presence of fruits (endogenous GA) during floral induction led us to propose that GA inhibits apple flowering by regulating expression of *MdTFL1* in the shoot apex (Fig. 27). Recent studies in rose and strawberry have provided valuable evidence for GA's role in flowering involving *TFL1* (Iwata et al. 2012; Randoux et al. 2012). Some rose and woodland strawberry varieties are annual (continuous-flowering); whereas some are perennial (once-flowering). It is found that these two different flowering behaviors are caused by mutations at the *TFL1* locus in both rose and woodland strawberry (Iwata et al. 2012). In rose, the annual plants are homozygous for the *TFL1* allele with a retrotransposon inserted in the second intron; and no full-length *TFL1* is not detectable due to the insertion. Similarly, in woodland strawberry, annual plants are homozygous for a *TFL1* with a 2-bp deletion in the first exon, leading to non-functional *TFL1*. Plants with

at least one functional *TFL1* allele are perennial, and therefore, this continuous-flowering phenotype is a recessive trait. Annual and perennial roses have different responses to exogenous GA. GA represses flowering and induces expression of *TFL1* in perennial roses but does not affect flowering in the annual roses (Randoux et al. 2012). The rose *TFL1* promoter contained GA-responsive elements, and plants with the elements deleted were hyposensitive to GA, suggesting the rose *TFL1* is a direct player in the repression of flowering by GA. In contrast, due to lack of a functional copy of *TFL1*, flowering of the annual roses was not affected by GA. Unlike *FT*-like genes, *TFL1*-like genes in the Rosaceae family appear to have originated from a single ancestral gene (Mimida et al. 2012). Therefore, *TFL1* is a very promising candidate for GA's repressive role in flowering in apple. Generating apple trees containing mutations in *MdTFL1* genes, using natural tfl1 mutant apple trees, or a genome-wide association study on the *TFL1* loci across apple varieties with different flowering responses to GA could help reveal the mystery.

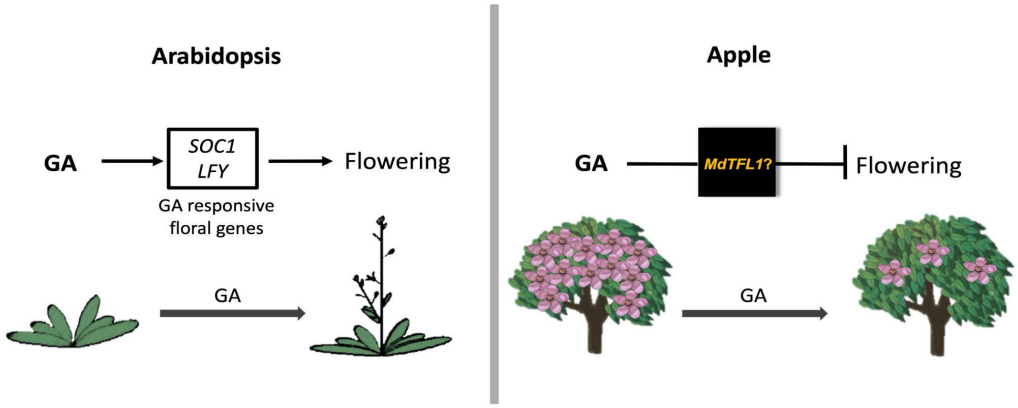

Figure 27. GA's paradoxical roles in flowering in *Arabidopsis thaliana* and apple. In the annual plant Arabidopsis, GA promotes flowering (specifically the transition from vegetative to reproductive phase) by upregulating GA-responsive floral genes such as *SOC1* and *LFY*. In contrast, spray of exogenous GA or the presence of fruits - a major source of endogenous GA - usually represses floral induction in apple, leading to reduced return bloom the following year. The identity of GA-responsive floral genes in apple is unknown. In the present study, we found that two copies of a previously reported floral repressor gene, *TFL1*, are promising candidates for the role of linking GA with repression of flowering in apple.

GA2ox genes stood out in our initial analyses due to their strong, persistent upregulation by GA (Chapter 2). They encode a group of enzymes that play a predominant role in GA deactivation. Expression of *GA2ox* genes is under tight regulation by both internal and external signals. First, *GA2ox* genes are under GA feedforward regulation: high levels of GA induce their expression, whereas low levels of GA repress it, ultimately leading to GA homeostasis. The spatial and developmental expression of *GA2ox* genes along with phenotypes conferred by loss and/overexpression of individual genes have provided tremendous opportunities to elucidate GA's functions in model plants, such as *AtGA2ox1* in hypocotyl elongation, *AtGA2ox2* in flower development, *AtGA2ox4* in flowering, and *AtGA2ox6* in embryo and nectar development (Xu et al. 2017; Rieu et al. 2008; Wang et al. 2004; Wiesen et al. 2016). Second, *GA2ox* genes are an integrator between GA signaling and other hormonal signaling pathways such as GA-cytokinin interaction through direct regulation of *ZmGA2ox1* in the shoot apical meristem maintenance in *Zea maize* and GA-ABA (abscisic acid) crosstalk through direct regulation of *AtGA2ox7*. Last but not the least, increasing evidence has shown that GA-mediated response to external signals including light, touch and abiotic stress is mainly through regulation of *GA2ox* genes (Colebrook et al. 2014), for example, *AtGA2ox2* in light-induced seed germination, *AtGA2ox7* in response to touch and salt, *SlGA2ox7* in GA-mediated stomatal closure induced by drought, and *AtGA2ox9* in cold response (Oh et al. 2006; Magome et al. 2008; Lange and Lange 2015; Lange et al. 2020; Shohat et al. 2021). It prompted us to develop regulation of *GA2ox* as a model to better understand GA's roles in apple flowering, which was another goal of my dissertation (Chapter 3).

Recent studies on evolution of GA metabolism and signaling have provided significant insights into GA biology from a unique perspective (Yoshida et al. 2020; Hernández-García et al. 2021). GAs appear to be present in all vascular plants. Genome-wide analysis of genes encoding the key components in GA metabolism and signaling revealed that they are widely present in higher plants, suggesting conserved pathways. We identified 16 copies of canonical *GA2ox*-like genes in the diploid 'Gala' apple genome, and they represent eight pairs of duplicated genes (Chapter 3). Most of these genes are under tight regulation by GA (GA feedforward regulation). To assess in which organs or structures these enzymes might act and to help elucidate the function of each gene, we analyzed their spatial and developmental expression profiles. Like their homologs in other plant species, these genes are widely spatially and developmentally expressed throughout the plant. RT-PCR-based expression patterns did not indicate apparent highly specific functions for any of the genes, as at least half of the genes were found to be expressed in every structure examined (except for fruit flesh). Even though overlapping functions between members of homoeologous pairs are anticipated, higher-resolution approaches such as reporter systems and *in situ* hybridization should help to narrow down their specialized expression sites and thus reveal any unique functions.

Apple trees have a long juvenile period (5 to 12 years from seeds germination to the first flowering), which has significantly hindered molecular and genetic studies in apple. *Arabidopsis thaliana* has been widely used as a model for eudicots. Even though Arabidopsis and apple are not phylogenetically closely related plant species, many components in GA metabolism and signaling are conserved and understanding of Arabidopsis *GA2ox* genes will provide valuable insights in the function of *GA2ox* in apple.

To develop regulation of *GA2ox* as a model in Arabidopsis, we generated a toolkit for *GA2ox* expression and function studies (Chapter 4). Using this toolkit, we confirmed the role of *AtGA2ox2* in flower and fruit development and many other processes. Ubiquitous expression of *AtGA2ox2* reminds us of one of the apple *GA2ox* genes, *MdGA2ox1B*, which was detected multiple times more than other genes in almost every apple structure examined. *MdGA2ox1B* is a close homolog of *AtGA2ox1*, *-2* and *-3*, and thus based on our new understanding of *AtGA2ox2*, we anticipate that *MdGA2ox1B* play numerous roles in apple growth and development as well. In addition, we found that two pairs of apple *GA2ox* genes, *GA2ox1A/1B* and *GA2ox2A/2B* were expressed in the shoot apex and inducible by both exogenous and presumable endogenous GA (Fig. 28). Three Arabidopsis genes, *AtGA2ox2*, *-4* and *-6* are expressed in the shoot apex. According to our and others' studies, *AtGA2ox4* is a major player in flowering among three genes, and loss of *AtGA2ox2* also affected flowering behavior. Thus, it is tempting for us to propose for future studies that two apple *GA2ox* pairs are involved in GA's role in flowering.

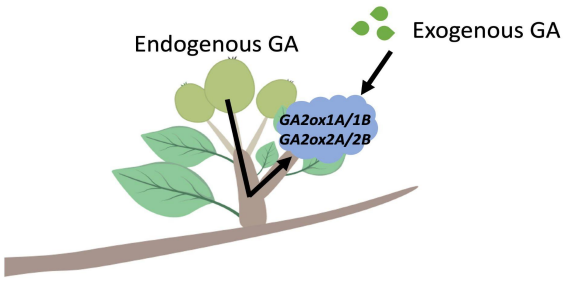

Figure 28. Expression of a subset of apple *GA2ox* genes was responsive to both endogenous and exogenous GA in the shoot apex in floral induction.

Decades of spray experiments in apple and many other tree fruits have significantly advanced our understanding of GA's effects on plant growth and development, and GA has been widely used as a plant growth regulator in agriculture and horticulture. However, many mysteries involving GA, such as GA's repressive role in flowering, alternate bearing, and unknown identity of genetic loci that are important for improved GA-associated traits, require thorough and deep understanding at the molecular and genetic levels. Only when the underlying mechanisms are unveiled, will the problems be solved and cultivars with desirable traits developed. In the era of rapid growth of genomics and bioinformatics, genomes of many apple varieties and tissues have been sequenced and readily available to use (Daccord et al. 2017; Sun et al. 2020). Leveraging these omics tools and the collection of apple germplasm is a promising approach to complicated scientific questions. Another approach would involve a combination of GA visualization and use of loss-of-function mutants and overexpression lines. A recent study reported the development of a GA biosensor, GIBBERELLIN PERCEPTION SENSOR 1 (GPS1), which senses nanomolar levels of bioactive GAs and enables high-resolution visualization of GA gradients in plants (Rizza et al. 2017). Transformation- and tissue culture-based approaches may be time-consuming, but they will bring us with an unprecedented understanding of GA biology in the long run.

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