A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Molecular Mechanisms Mediating Gibberellin-Induced Parthenocarpic Fruit Development in Grapevines (*Vitis* spp.)

지베렐린 처리에 의해 유도되는 포도 단위결과의 분자생물학적 작용 기작 구명

BY

YOUN YOUNG HUR

AUGUST, 2014

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UNDER THE DIRECTION OF DR. HEE JAE LEE SUBMITTED TO THE FACULTY OF

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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DEPARTMENT OF HORTICULTURAL SCIENCE AND BIOTECHNOLOGY

JUNE, 2014

APPROVED AS A QUALIFIED DISSERTATION OF YOUN YOUNG HUR

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Molecular Mechanisms Mediating Gibberellin-Induced Parthenocarpic Fruit Development in Grapevines (*Vitis* spp.)

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ABSTRACT

The concept that gibberellin (GA) application on seeded grapevines induces parthenocarpy has been known for decades in viticulture, but the molecular mechanism underlying this induction is poorly understood. GA was applied to inflorescence clusters of seeded diploid grapevine cultivar 'Tamnara' (*Vitis* spp.) at 14 days before full bloom (DBF). Morphological and molecular effects of GA application were examined on the induction of parthenocarpic fruit development. With GA application, ovaries were enlarged and pollen tube growth was completely inhibited. *Vitis* GA oxidases, the key determinants for GA level, were characterized through phylogenetic analysis with *Arabidopsis* GA oxidase. Five VvGA 20-oxidase (VvGA20ox), three VvGA 3-oxidase (VvGA3ox), and nine VvGA 2-oxidase (VvGA2ox) family proteins, and one VvGA methyltransferase (VvGAMT)

and one *Vitis* cytochrome P450 (VvCYP) 714A proteins were identified, and their expression patterns were analyzed during inflorescence development from 14 DBF to 5 days after full bloom (DAF). *VvGA2ox1*, *VvGA20ox3*, and *VvGA3ox2* transcripts were most abundantly expressed in each gene family at 7, 5, and 2 DBF, respectively. Following GA application at 14 DBF inducing seedlessness, GA catabolic genes such as *VvGAMT2, VvGA2ox3*, and *VvGA2ox4* were up-regulated at 12 DBF, full bloom, and 5 DAF, respectively. Conversely, GA biosynthetic genes, *VvGA20oxs* and *VvGA3oxs*, were down-regulated at near full bloom, and the timing of their peak expression was changed. These results suggest that GA application at pre-bloom changes the GA biosynthesis into GA catabolic pathway at near full bloom by altering the transcript level and timing of GA oxidase genes expression during grapevine inflorescence development. In addition, the transcriptional levels of the putative negative regulators of fruit set initiation, including *Vitis auxin/indole-3-acetic acid transcription factor 9* (*VvIAA9*), *Vitis auxin response factor 7* (*VvARF7*), and *VvARF8* were also monitored during inflorescence development. Without GA application, *VvIAA9, VvARF7*, and *VvARF8* were expressed at a relatively high level before full bloom, but decreased thereafter following pollination. However, after GA application at 14 DBF, the expression levels of *VvIAA9* and *VvARF7* declined at 5 DBF prior to pollination. The effects of GA application on auxin levels or auxin signaling were also analyzed by monitoring the expression patterns of auxin-biosynthetic and -responsive genes with or without GA application. Transcript levels of the auxin biosynthetic genes of *Vitis anthranilate synthase β subunit* (*VvASB1-like*),

Vitis YUCCA2 (*VvYUC2*), and *VvYUC6* were not significantly changed by GA application. However, the expressions of *Vitis Gretchen Hagen3.2* (*VvGH3.2*) and *VvGH3.3*, auxin-responsive genes, were up-regulated from 2 DBF to full bloom with GA application. Furthermore, the *Vitis* GA signaling gene, *VvDELLA* was up-regulated by GA application during 12 to 7 DBF, prior to down-regulation of *VvIAA9* and *VvARF7*. These results suggest that *VvIAA9* and *VvARF7* are negative regulators of fruit set initiation in grapevines, and GA signaling is integrated with auxin signaling via *VvDELLA* during parthenocarpic fruit development in grapevines.

Key words: Auxin, Auxin response factor, Gibberellin, Gibberellin methyltransferase, Gibberellin oxidase, Grapevine inflorescence, Parthenocarpy, Seedlessness

Student Number: 2009-30292

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ABBREVIATIONS

ARF	auxin response factor
ASB	anthranilate synthase β subunit
Aux/IAA	auxin/indole-3-acetic acid
AuxREs	auxin response elements
CPS	ent-copalyl diphosphate synthase
CYP	cytochrome P450
DAF	days after full bloom
DBF	days before full bloom
GA	gibberellin
GAMT	gibberellin methyltransferase
GH3	*Gretchen Hagen3*
KAO	ent-kaurenoic acid oxidase
KO	ent-kaurene oxidase
KS	ent-kaurene synthase
NLS	nuclear localization signal
ODD	oxoglutarate-dependent dioxygenases
PIN	PIN-FORMED

GENERAL INTRODUCTION

Grape species are one of the oldest cultivated fruit species in the world (Pelsy et al., 2010). They belong to the genus *Vitis* in the Vitaceae family, commonly called the grape family (The Angiosperm Phylogeny Group, 2009). The genus *Vitis* consists of approximately 60 species; approximately 40 species of these are distributed throughout north America, and approximately 20 species are located in central and east Asia (Aradhya et al., 2003). *V. vinifera* originated from the Turkish near east; it accounts for more than 95% of the world *Vitis* crops (Arroyo-García et al., 2006). Interspecific hybrid cultivars have been selected from crosses of *V. vinifera* with other species, including *V. labrusca*, *V. amurensis*, *V. riparia*, *V. rupestris*, and *V. aestivalis*. These hybrid cultivars are important, locally, but are minor components of world viticulture and enology (Reisch et al., 2012).

Seedlessness is one of the most desirable traits of table and raisin grapes. Seedless grapes are most commonly produced by planting the two types of genetically seedless cultivars that exist in grapevines (Ledbetter and Ramming, 1989). The first type is the ‘Corinth’ cultivar, in which seedlessness is caused by parthenocarpy. ‘Corinth’ berries are small and spherical and are therefore only used to produce raisins. The second type of seedlessness is caused by stenospermocarpy (Ledbetter and Ramming, 1989). In stenospermocarpic berries, pollination and

fertilization occur normally, but the seed coat and endosperm cease development at early stages, leaving only undeveloped seeds or seed traces in the mature berries (Ledbetter and Burgos, 1994; Ledbetter and Ramming, 1989). Stenospermocarpic cultivars have been used to introduce seedlessness into breeding programs, and they have relatively large fruit (Ledbetter and Burgos 1994, Park et al., 2003). The stenospermocarpic *V. vinifera* cultivars include 'Thompson Seedless', 'Ruby Seedless', 'Cheongsoo', and 'Flame Seedless'. This species is not particularly suited to the Korean climate, with hot and humid summers and cold and dry winters. In this climate, the stenospermocarpic *V. vinifera* cultivars are prone to disease and insect infestation, have reduced cold tolerance and greater susceptibility to freezing damage, and are difficult to be harvested.

Alternatively, seedlessness can be induced by the application of gibberellins (GAs) to the seeded cultivars at strategic time windows during flower and fruit development. This method has been used to produce seedless grapes since the 1960s (Dass and Randhawa, 1968; Lu et al., 1997). A seedless grape cultivation techniques employing GA application have been used for the production of *V. labruscana* (*V. vinifera* × *V. labrusca*) table grapes in Japan and Taiwan, which have similar climate conditions as those in Korea. The production of seedless grapes using GA is very well established. Seedless grapes can be produced through a two-step GA application process, which usually involves one GA application

before flowering and the other GA application after flowering. The first GA application induces seedlessness, which the second application causes berry enlargement. Although GA-mediated parthenocarpy is a highly desirable trait for table grapes, the mechanism of GA-induced parthenocarpic fruit development has not been completely understood.

In this study, molecular mechanisms underlying the induction of parthenocarpic fruit development were investigated in the seeded cultivar 'Tamnara' grapevines following GA application. Genes for *Vitis* GA metabolic enzymes were identified and their expression changes were monitored during inflorescence development with and without GA application. Changes in the expression levels of the genes involved in fruit set, *Vitis auxin/indole-3-acetic acid 9*, *Vitis Auxin Response Factor 7*, and *Vitis DELLA*, were monitored to determine whether GA application coordinates auxin signaling during parthenocarpic fruit development.

LITERATURE REVIEW

Gibberellin metabolism

The diterpenoid GAs were first identified from the pathogenic fungus *Gibberella fujikuroi*, which is the causal agent of the foolish-seedling disease of rice that results in excessive elongation of infected plants (Yabuta and Sumiki, 1938). More than 130 GAs have been identified in plants, fungi, and bacteria, but not many are biologically active (Yamaguchi, 2008). Many non-bioactive GAs exist in plants, and these serve as precursors for the bioactive forms or are de-activated catabolites. The major bioactive GAs include GA_1, GA_3, GA_4, and GA_7; and they are derived from a basic diterpenoid carboxylic acid skeleton; and share a common C3-hydroxyl group (Yamaguchi, 2008).

Enzymes involved in GA metabolism

GAs are biosynthesized in the following three stages by the sequential activities of several classes of enzymes (Hedden and Phillips, 2000; Hedden and Thomas, 2012; Sakamoto et al., 2004; Sun, 2008; Yamaguchi, 2008): (1) conversion of geranylgeranyl diphosphate to *ent*-kaurene by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) in the plastid; (2) sequential modification of *ent*-kaurene by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO) in the endoplasmic reticulum, to ultimately produce GA_{12}; and (3) biosynthesis of

active GAs in the cytoplasm via flux through two parallel pathways involving 13-hydroxylation and non-13-hydroxylation, and consecutive oxidations of C-20 and C-3 of GA_{12} and GA_{53} by 2-oxoglutarate-dependent dioxygenases (2ODDs), GA 20-oxidases (GA20oxs), and GA 3-oxidases (GA3oxs). These bioactive GAs undergo three alternative deactivation reactions to produce non-bioactive catabolites. GA2ox, a 2ODD member, introduces a 2β-hydroxyl group to deactivate bioactive GAs. GA methyltransferase (GAMT) methylates the bioactive GAs to produce non-bioactive GA catabolites (Varbanova et al., 2007). Cytochrome P450 monooxygenase 714A oxidizes the bioactive GAs into 16α and 17-epoxidation forms (Zhang et al., 2011).

While early stage enzymes in the GA biosynthesis pathway such as CPS, KS, KO, and KAO are encoded by single genes (Helliwell et al., 1998; Sun and Kamiya, 1994; Yamaguchi et al., 1998), GAoxs that function at later stages of GA biosynthesis are encoded by gene families in higher plants (Hedden and Phillips, 2000; Sakamoto et al., 2004; Song et al., 2011; Yamaguchi, 2008). Although null mutants of CPS, KS, and KO, known as *ga1*, *ga2*, and *ga3*, respectively, showed GA-deficient dwarf phenotypes, bioactive GA levels were not increased by the overexpression of *AtCPS* or *AtKS* (Fleet et al., 2003). While *ga4* and *ga5* null mutants of *GA3ox1* and *GA20ox1*, respectively, were not associated with severe GA-deficient phenotypes due to the compensation effects by the same family genes (Chiang et al., 1995; Phillips et al., 1995), bioactive

GA levels were increased by the overexpression of these 2ODDs (Coles et al., 1999; Itoh et al., 1999). Expression levels of the GA catabolic genes *GA2oxs*, *GAMTs*, and CYP714A also affected bioactive GA levels as shown in their mutants and in overexpressing transgenic plants (Schomburg et al., 2003; Varbanova et al., 2007, Zhang et al., 2011), implying that bioactive GA levels are determined by these multigene-encoded GAoxs in plants.

GA homeostasis

Bioactive GA levels have been reported to be modulated via feedback regulation by GA metabolic genes and enzymes (Hedden and Phillips, 2000; Hedden and Thomas, 2012; Olszewski et al., 2002; Yamaguchi, 2008). Application of exogenous GA down-regulated GA biosynthetic genes and up-regulated GA catabolic genes in *Arabidopsis* and tobacco (Gallego-Giraldo et al., 2008; Rieu et al., 2008; Thomas et al., 1999). Feedback regulation was not adapted to all of the GA metabolic genes, only to the mainly expressed *GAoxs* (Hedden and Thomas, 2012; Sun, 2008). *AtGAMT1* and *AtGAMT2* affected endogenous GA levels in *Arabidopsis*, but whether they are controlled by feedback regulation is unknown (Varbanova et al., 2007).

Hormones and parthenocarpic fruit development

Fruit set is initiated only after two sequential events, pollination and

fertilization (Gillaspy et al., 1993), with changes in the levels of endogenous plant hormones, primarily GAs and auxins (Coombe, 1960; Kuhn and Arce-Johnson, 2012; Ozga et al., 2003; Wang et al., 1993). Application of GA or auxin can trigger fruit set even without pollination and can induce parthenocarpic fruit development (de Jong et al., 2009a; Gustafson, 1936; Schwabe and Mills, 1981; Vivian-Smith and Koltunow, 1999; Wittwer et al., 1957).

Auxin signaling genes related to parthenocarpic fruit development

Although the molecular mechanisms by which either GA or auxin mediates fruit set initiation are not clearly established, several auxin signaling genes related to parthenocarpic fruit development have been identified. Auxin/indole-3-acetic acid (Aux/IAA) transcription factor family are known to be essential repressors for auxin signaling in various developmental processes including fruit set (Ulmasov et al., 1997). For example, down-regulation of Aux/IAA9 has been shown to induce parthenocarpic fruit development in tomato (Wang et al., 2005). Two auxin response factors (ARF) related to parthenocarpic fruit development have also been identified in *Arabidopsis* (Goetz et al., 2006; 2007) and tomato (de Jong et al., 2009b). ARF family act as regulators of auxin-responsive genes by specifically binding to auxin response elements in the promoters of auxin-responsive genes. ARFs play important roles in diverse developmental processes in embryos, hypocotyls, floral organs, and fruit

(Finet et al., 2010; Goetz et al., 2006; Hardtke and Berleth, 1998; Harper et al., 2000; Li et al., 2004; Li et al., 2006; Schruff et al., 2006). The *Arabidopsis fruit without fertilization* (*fwf*) mutant produced parthenocarpic fruit as the result of the expression of truncated *ARF8* (Goetz et al., 2006; 2007), and the same mutation in the *SlARF8* in tomato also induced parthenocarpy (Goetz et al., 2007). Parthenocarpy induction was also observed in a mutant that silences *ARF7* in tomato (de Jong et al., 2011). The expression of *SlARF7* was maintained at high levels before pollination and rapidly declined after pollination due to increased auxin content (Coombe, 1960; de Jong et al., 2009a; Gillaspy et al., 1993; Kuhn and Arce-Johnson, 2012), in a manner similar to its down-regulation upon exogenous auxin application (de Jong et al., 2009b). Since parthenocarpy induction resulted from the disruption of high expression levels of *Aux/IAA9*, *ARF7*, and *ARF8*, these genes are regarded as negative regulators of fruit set initiation.

Auxin efflux also plays a role in fruit set initiation, which is regulated by the PIN-FORMED (PIN) protein family. PIN proteins maintain an asymmetrical auxin distribution, which is important for auxin signaling in diverse developmental processes (Křeček et al., 2009; Pattison and Catala, 2012; Vanneste and Friml, 2009; Zažímalova et al., 2007), including reproductive organ development (Sundberg and Østergaard, 2009). Considering parthenocarpy induction either by auxin transport inhibitor (Serrani et al., 2010) or the silencing of tomato PIN-FORMED 4

(*SlPIN4*) (Mounet et al., 2012), auxin transport by PIN proteins is also crucial for fruit set initiation.

GA biosynthesis and signaling during parthenocarpyic fruit development

Parthenocarpic fruit development has been observed in tomato mutants (*pat*, *pat-2*, and *pat-3/pat*-4) that overexpress GA biosynthesis genes (Fos et al., 2000, 2001; Olimpieri et al., 2007). GA signaling during parthenocarpic fruit development was demonstrated in *DELLA* silencing mutants in *Arabidopsis* (Fuentes et al., 2012) and tomato (Marti et al., 2007). The roles of DELLA protein in *ARF7* expression and partial auxin signaling during parthenocarpic fruit development were demonstrated in the *procera* (*pro*) mutant of *DELLA* in tomato (Carrera et al., 2012).

Integration of GA and auxin signaling

Auxin transport controls organ initiation, development, and tropic responses. Auxins are synthesized in the shoot apex and transported toward the root tip via the PIN auxin efflux carriers. When the shoot is removed, thus removing the shoot-derived auxin source, roots stop growing and fail to respond normally to GA. This reduced GA-responsiveness can be suppressed when IAA is applied to the site of shoot removal. Genetic and cellular biological analyses showed that root-growth cessation correlates with the accumulation of DELLAs in the root

and is suppressed in *DELLA* loss-of-function mutants (Fu and Harberd, 2003). Thus, auxin and auxin transport may control DELLA abundance and root growth. Conversely, GAs have been reported to control auxin transport and PIN protein abundance (Willige et al., 2011). GAs promote degradation of the auxin efflux carriers PIN1 and PIN2 in root tips and inflorescence stems, possibly by promoting targeting of these transporter proteins for degradation in the vacuole (Vieten et al., 2007; Willige et al., 2011). At the physiological level, PIN proteins are down-regulated in GA mutants and GA-signaling mutants, and this down-regulation correlates with reduced auxin transport in inflorescence stems, defective embryo development, and reduced root gravitropism.

Crosstalk between GA and auxin during fruit set initiation has been demonstrated by the observed effects of auxin on GA biosynthesis (Dorcey et al., 2009; Serrani et al., 2008) and the partial involvement of GA signaling in silencing mutants of *ARF7* (de Jong et al., 2009b, 2011) and *PIN4* (Mounet et al., 2012). Using the *pro* loss-of-function mutant of *SlDELLA*, Carrera et al. (2012) demonstrated that GA affected auxin signaling by down-regulating *SlIAA9* and *SlARF7*, which are negative regulators of fruit set initiation. Thus, GA and auxin signaling pathways may mutually activate each other during fruit set initiation, and this interaction appears to be associated with the induction of parthenocarpic fruit development.

Use of GA in viticulture

Gibberellin is involved in multiple aspects of growth and development, including seed maturation and germination, stem elongation, and floral induction (Hedden and Kamiya, 1997; Olszewski et al., 2002; Richards et al., 2001). GA is used to induce seedlessness in seeded grapevine cultivars, and improves fruit set, rachis elongation, and berry enlargement (Coombe, 1960; Dass and Randhawa, 1968; Dokoozlian and Peacock, 2001; Giacomelli et al., 2013; Gustafson, 1936; Iwahori et al., 1968; Lu et al., 1997).

Induction of seedlessness in seeded cultivars

GA has been used to produce seedless grapes since the 1960s (Dass and Randhawa, 1968; Lu et al., 1997). Seedless grapes are produced by applying exogenous GA at two developmental stages, before flowering and again after flowering. The first application induces seedlessness and the second enlarges the developing berries. GA application inhibits pollen tube growth in the Delaware grapevine cultivar (Okamoto and Miura, 2005), without causing any pistil defects. The number of cell layers in the pistil transmitting tract was not affected by exogenous GA (Okamoto and Miura, 2005). Although the physiological effects induced by the timing of GA application on grapevine cultivars have been documented (Dass and Randhawa, 1968; Lu et al., 1997; Okamoto and Miura, 2005), the molecular mechanisms involved in GA-induced seedlessness are unknown.

Improving fruit set

The process of fruit set in grapevines is one of the most crucial events in the annual production of quality clusters. Fruit set is initiated only after the two sequential events of pollination and fertilization (Gillaspy et al., 1993). Fruit set is affected by planting methods, vineyard management, and climatic conditions during flowering. Unfertilized flowers are abscised from the plant. However, GA application suppresses development of the abscission layer at the base of the pedicel so that the unfertilized flower is not shed. The molecular mechanisms involved in hormonal regulation of fruit set initiation have been studied (de Jong et al., 2009a; Vivian-Smith and Koltunow, 1999). Although GA and auxin biosynthesis genes were up-regulated after pollination in grapevines (Dauelsberg et al., 2011; Kuhn and Arce-Johnson, 2012), the question of how GA mediates fruit set in unfertilized flowers has not been answered unequivocally.

Rachis elongation and berry enlargement

Fruit cluster morphology and berry size are economically important traits for grapevine cultivars. These traits are often controlled by strategic applications of exogenous GA (Dokoozlian and Peacock, 2001; Khurshid et al., 1992; Weaver, 1961). In wine grape cultivars, GA application is performed at anthesis to achieve loosening and better aeration of the clusters, which renders them less susceptible to fungal rot. In table grape cultivars, GA application is often performed after fruit set to increase berry size (Giacomelli et al., 2013). Growth hormone production is limited in seedless grapevine cultivars, which limits cell expansion and berry size

(Coombe, 1960; Nitsch et al., 1960). GA sprays are often applied to seedless cultivars after fruit set to increase berry size. GA application also reduces cluster compactness by promoting rachis elongation.

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CHAPTER 1

Transcriptional Changes of Gibberellin Oxidase Genes in Grapevines with or without Gibberellin Application during Inflorescence Development

ABSTRACT

The concept that gibberellin (GA) application on seeded grapevines induces seedlessness has been known for decades in viticulture. GA was applied to inflorescence clusters of seeded diploid grapevine cultivar 'Tamnara' (*Vitis* spp.) at 14 days before full bloom (DBF). Morphological and molecular effects of GA application were examined on the induction of parthenocarpic fruit development. With GA application, ovaries were enlarged and pollen tube growth was completely inhibited. *Vitis* GA oxidase enzymes, key determinants for GA level, were characterized through phylogenetic analysis with *Arabidopsis* GA oxidase enzymes. Five VvGA 20-oxidase (VvGA20ox), three VvGA 3-oxidase (VvGA3ox), and nine VvGA 2-oxidase (VvGA2ox) family proteins, and one VvGA methyltransferase (VvGAMT) and one *Vitis* cytochrome P450 (VvCYP) 714A proteins were identified, and their expression patterns were analyzed during inflorescence development from 14 DBF to 5 days after full bloom (DAF). *VvGA2ox1*, *VvGA20ox3*, and *VvGA3ox2* were the most abundantly

expressed genes in each gene family at 7, 5, and 2 DBF, respectively. Following GA application at 14 DBF inducing seedlessness, GA catabolic genes such as *VvGAMT2*, *VvGA2ox3*, and *VvGA2ox4* were up-regulated at 12 DBF, full bloom, and 5 DAF, respectively. Conversely, most GA biosynthetic genes, *VvGA20oxs* and *VvGA3oxs*, were down-regulated at near full bloom, and the timing of their peak expression was changed. These results suggest that GA application at pre-bloom changes the GA biosynthesis into GA catabolic pathway at near full bloom by altering the transcription level and timing of GA oxidase genes during grapevine inflorescence development.

INTRODUCTION

Gibberellins (GAs) involve in multiple aspects of growth and development, including seed maturation and germination, stem elongation, and floral induction (Hedden and Kamiya, 1997; Olszewski et al., 2002; Richards et al., 2001). Endogenous GA levels in inflorescence increase only after pollination and thereby cause fruit set initiation and development (de Jong et al., 2009a; Gillaspy et al., 1993). During inflorescence and fruit development, especially, the alteration of GA metabolism causes delay in floral development (Wilson et al., 1992), inhibition of stamen and pollen biogenesis (Goto and Pharis, 1999; Hu et al., 2008; Plackett et al., 2011), inhibition of pollen tube growth (Chhun et al., 2007; Singh et al., 2002), seed abortion (Singh et al., 2002), and seedless fruit development (de Jong et al., 2009a). Application of GA to the inflorescence can induce fruit set initiation without pollination, parthenocarpy (de Jong et al., 2009a; Vivian-Smith and Koltunow, 1999). The regulation of GA levels during inflorescence development is critical for fruit set initiation and fruit development.

Although more than 130 GAs were identified from plants, fungi, and bacteria (http://www.plant-hormones.info/gibberellin_nomenclature.htm), only GA_1, GA_3, GA_4, and GA_7 have been identified as bioactive forms in plants (Hedden and Phillips, 2000; MacMillan, 2002). GAs are biosynthesized by the actions of several classes of enzymes (Hedden and Thomas, 2012; Sakamoto et al., 2004; Sun, 2008; Yamaguchi, 2008) in three stages: (1) conversion of geranylgeranyl diphosphate to *ent*-kaurene

by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS); (2) production of GA_{12} from *ent*-kaurene after sequential modifications by *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO); and (3) biosynthesis of active GAs derived after 13-hydroxylation or non-13-hydroxylation with consecutive oxidations of GA_{12} and GA_{53} by 2-oxoglutarate-dependent dioxygenases (2ODDs), GA 20-oxidases (GA20oxs) and GA 3-oxidases (GA3oxs), respectively. These bioactive GAs are deactivated by another 2ODD, GA2ox, GA methyltransferase (GAMT), and cytochrome P450 monooxygenase 714A (CYP714A), introducing a 2β-hydroxyl group, methylation (Varbanova et al. 2007), and 16α, 17-epoxidation (Zhang et al., 2011), respectively. GAoxs that function at later stages of GA metabolism are encoded by gene families in higher plants (García-Hurtado et al., 2012; Hedden and Phillips, 2000; Sakamoto et al., 2004; Serrani et al., 2007; Song et al., 2011; Yamaguchi, 2008), and they have been reported to modulate bioactive GA levels via feedback regulation (Hedden and Phillips, 2000; Hedden and Thomas, 2012; Olszewski et al., 2002; Yamaguchi, 2008), with the differential expression of each gene depending on the developmental stage and tissue (Mitchum et al., 2006; Phillips et al., 1995; Rieu et al., 2008; Yamaguchi et al., 2001).

In grapevines, the effects of GA have long been studied on ripening, berry enlargement, and seedlessness induction in seeded cultivars (Coombe, 1960; Dass and Randhawa, 1968; Gustafson, 1936; Iwahori et al., 1968; Lu et al., 1997). GA has been applied for seedless grape production since the 1960s (Dass and Randhawa, 1968; Lu et al., 1997). Seedless grapes could be produced with two step GA application. The two

step GA application has usually been employed before flowering and again after flowering; one for inducing seedlessness and the other for berry enlargement. To understand how GA affects grapevine ripening and berry enlargement, transcriptomic and proteomic analyses have been attempted (Deluc et al., 2007; Grimplet et al., 2007; Lijavetzky et al., 2012; Mathieu et al., 2005; Wang et al., 2012). However, molecular mechanisms underlying GA-induced seedlessness remain unknown except in the context of GA application timing effects on certain cultivars (Dass and Randhawa, 1968; Lu et al., 1997; Okamoto and Miura, 2005). In this study, *Vitis* GA metabolic enzymes, in particular, GAoxs, GAMT2, and CYP714A1, which are key metabolic determinants modulating GA level, were investigated and the expression levels of these genes were monitored during grapevine inflorescence development. Transcriptional changes in these *Vitis* GA metabolic genes were also analyzed upon GA application to induce seedlessness.

MATERIALS AND METHODS

Plant material and GA application

Five-year-old grapevines of seeded diploid cultivar ‘Tamnara’ (*Vitis* spp.) grown in an overhead arbor system were used for GA application and gene expression analysis. The cultivar used in this study was bred from the cross between ‘Campbell Early’ (*V. labruscana*) and ‘Himrod’ (*Vitis* spp.) at the National Institute of Horticultural and Herbal Science, Suwon, Republic of Korea in 1998 (Park et al., 2004). A GA solution (Dongbu, Seoul, Korea) at 100 ppm was applied as described by Okamoto and Miura (2005) onto inflorescence clusters at 14 days before full bloom (DBF), which were corresponded to the stage showing eight leaves separated and compact grouped flower, based on the E-L system of Coombe (1995), and they were labeled. The clusters were harvested at 0, 1, 2, 4, 7, 9, 12, 14, 16, and 19 days after the GA application. Harvested inflorescence samples were immediately frozen in liquid N_2 and stored at –80°C until used in the RNA extraction.

Histological analysis

Ovaries from inflorescence clusters applied with or without GA at 14 DBF were collected at full bloom and separately fixed in 2.5% glutaraldehyde in a solution of 0.1 M phosphate buffer (pH 7.2). After dehydration in an ethanol series, the ovary samples were embedded and

polymerized overnight in 100% Epon at 60°C. Semi-thin sections (1.5 μm thickness) were prepared using a microtome (Leica, Hubloch, Germany) and stained with periodic acid-Schiff reagent. Stained tissue sections were viewed with a light microscope (Axioskop 2, Carl Zeiss, Jena, Germany).

Flowers from inflorescence clusters applied with or without GA at 14 DBF were emasculated at 1 DBF and pollinated artificially at full bloom. At 48 h after artificial pollination, pollen tubes inside the pistils from inflorescences were stained according to the method of Hülskamp et al. (1995). The pistils were cleared in 10% chloral hydrate solution at 65°C for 5 min, and washed and softened with 5 M NaOH at 65°C for 5 min. After rinsing with tap water, the pistils were stained with 0.1% aniline blue in 0.1 M phosphate buffer (pH 8.3) in darkness for 3 h and briefly rinsed. The stained pistils were mounted and gently squashed for observation under a fluorescence microscope (LSM510 META, Carl Zeiss).

Protein identification

Amino acid sequences of *Arabidopsis* GA metabolic enzymes obtained from the GenBank of National Center for Biotechnology Information (NCBI) were used in a search of homologous grapevine proteins using the BLASTP algorithm downloaded from the NCBI as a local blast browser. Five AtGA20ox family proteins (GenBank accession numbers: NP_194272, NP_199994, NP_196337, NP_176294, and NP_175075) and four AtGA3ox family proteins (GenBank accession numbers: NP_173008,

NP_178150, NP_193900, and NP_178149) were used to identify *Vitis* orthologues in the local downloaded blast browser. *Arabidopsis* GA catabolic enzymes, seven AtGA2ox family proteins (GenBank accession numbers: NP_177965, NP_174296, NP_181002, NP_175233, NP_171742, NP_175509, and NP_193852), two AtGAMT family proteins (GenBank accession numbers: NP_194372 and NP_200441), and two AtCYP714A family proteins (GenBank accession numbers: NP_568463 and NP_197872) were also used to identify *Vitis* GA catabolic enzymes. Among the candidates, *Vitis* proteins with higher than 40% identity, adopted as a representation of functional similarity in searching for homologous genes (Sangar et al., 2007; Schuler and Werck-Reichhart, 2003), to their *Arabidopsis* counterparts were selected and used in phylogenetic analysis. VvCYP714A1 was grouped into a CYP714A subfamily according to Schuler and Werck-Reichhart (2003) and confirmed in Cytochrome P450 Homepage (http://drnelson.uthsc.edu/CytochromeP450.html). Gene sequences were identified in the Grape Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/), version 12X. The *Vitis* GA metabolic genes and their accession numbers are listed in Tables 1-1 and 1-2.

Phylogenetic analysis

Phylogenetic analyses were performed with the amino acid sequences of the *Vitis* GA metabolic enzymes identified in this study and with those of

Table 1-1. GA biosynthetic genes in grapevines.

Gene family	Gene name	GenBank accession No.[z]	Gene ID[y]	Chromosome
GA 20-oxidase	*VvGA20ox1*	CBI24470	GSVIVT01018453001	16
	VvGA20ox2	CBI38818	GSVIVT01027572001	19
	VvGA20ox3	CBI37510	GSVIVT01026453001	4
	VvGA20ox4	CBI18950	GSVIVT01008782001	18
	VvGA20ox5	CBI37521	GSVIVT01026466001	4
GA 3-oxidase	*VvGA3ox1*	CBI36201	GSVIVT01017173001	9
	VvGA3ox2	CBI36204	GSVIVT01017178001	9
	VvGA3ox3	CBI20992	GSVIVT01035796001	4

[z]GenBank accession numbers obtained from NCBI.

[y]Gene ID obtained from Grape Genome Browser.

Table 1-2. GA catabolic genes in grapevines.

Gene family	Gene name	GenBank accession No.[z]	Gene ID[y]	Chromosome
GA 2-oxidase	*VvGA2ox1*	CBI30887	GSVIVT01021468001	10
	VvGA2ox2	CBI33024	GSVIVT01000689001	19
	VvGA2ox3	CBI33023	GSVIVT01000687001	19
	VvGA2ox4	CBI22844	GSVIVT01034945001	5
	VvGA2ox6	CBI36990	GSVIVT01028169001	7
	VvGA2ox7A	CBI23932	GSVIVT01001966001	19
	VvGA2ox7B	CBI27476	GSVIVT01010228001	17
	VvGA2ox7C	CBI23931	GSVIVT01001965001	19
	VvGA2ox8	CBI23168	GSVIVT01012628001	10
GA methyltransferase	*VvGAMT2*	CBI16576	GSVIVT01025514001	6
16α,17-epoxidase	*VvCYP714A1*	XP_002266868	GSVIVT01032871001	13

[z]GenBank accession numbers obtained from NCBI.

[y]Gene ID obtained from Grape Genome Browser.

Arabidopsis using neighbor-joining algorithms of the MEGA5 program (Tamura et al., 2011). All ambiguous positions were removed for each sequence pair (pairwise deletion option), and bootstrap analysis using 1,000 replicates was performed to evaluate the reliability.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from the harvested inflorescence samples, including berries and pedicles at each developmental stage, using an RNA extraction method (Chang et al., 1993) modified to remove polysaccharides and phenolic compounds. For cDNA synthesis, a total of 0.5 μg RNA was reverse transcribed with 200 U Moloney murine leukemia virus reverse transcriptase (Takara, Tokyo, Japan) and an oligo dT primer, according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

qRT-PCR analysis was performed as described by Poupin et al. (2007) on an ABI 7500 Fast RealTime PCR system and 7500 system software, version 2.0.5 (Applied Biosystems, Lincoln, CA, USA) under universal thermal cycling conditions described by the manufacturer. The PCR primers used are shown in Supplementary Table 1.1. *Vitis Actin1* gene (*VvActin1*, GenBank accession number: AB372563) was used as an endogenous control for the normalization of gene expression. Cycle

threshold (C_T) values obtained were used to generate ΔC_T values ($C_{T\ target\ gene} - C_{T\ VvActin1}$). The relative expression of each gene was calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$) and normalized to that of samples from 14 DBF. To compare gene expression profiles, the hierarchical clustering of relative gene expression data was conducted using the Pearson correlation with complete merge method. Analysis of qRT-PCR efficiency showed that all amplicons of all genes used in this study were in optimal ranges of 95-105% (Fig. 1-1).

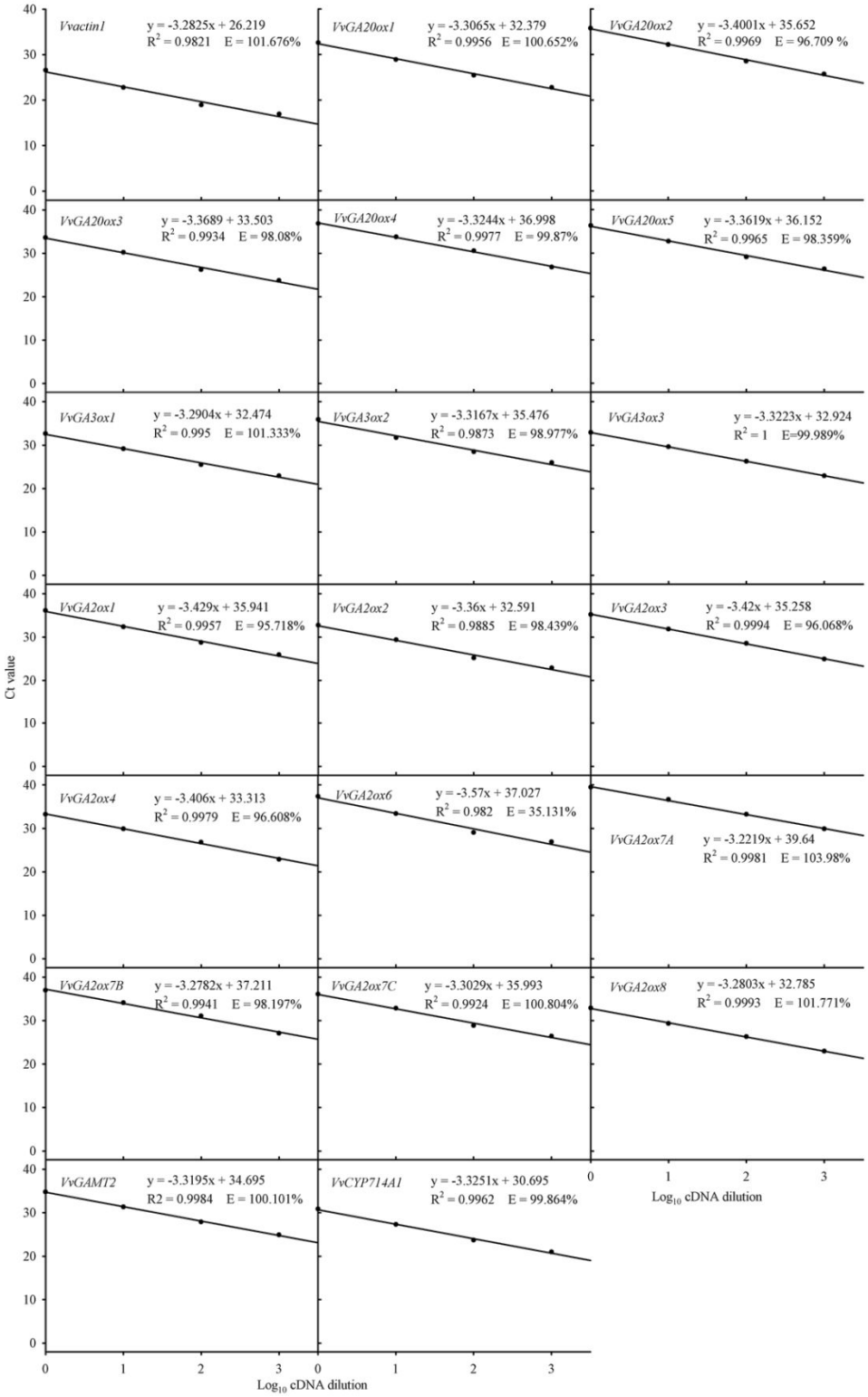
Vvactin1 y = -3.2825x + 26.219 R² = 0.9821 E = 101.676%
VvGA20ox1 y = -3.3065x + 32.379 R² = 0.9956 E = 100.652%
VvGA20ox2 y = -3.4001x + 35.652 R² = 0.9969 E = 96.709 %
VvGA20ox3 y = -3.3689 + 33.503 R² = 0.9934 E = 98.08%
VvGA20ox4 y = -3.3244x + 36.998 R² = 0.9977 E = 99.87%
VvGA20ox5 y = -3.3619x + 36.152 R² = 0.9965 E = 98.359%
VvGA3ox1 y = -3.2904x + 32.474 R² = 0.995 E = 101.333%
VvGA3ox2 y = -3.3167x + 35.476 R² = 0.9873 E = 98.977%
VvGA3ox3 y = -3.3223x + 32.924 R² = 1 E=99.989%
VvGA2ox1 y = -3.429x + 35.941 R² = 0.9957 E = 95.718%
VvGA2ox2 y = -3.36x + 32.591 R² = 0.9885 E = 98.439%
VvGA2ox3 y = -3.42x + 35.258 R² = 0.9994 E = 96.068%
VvGA2ox4 y = -3.406x + 33.313 R² = 0.9979 E = 96.608%
VvGA2ox6 y = -3.57x + 37.027 R² = 0.982 E = 35.131%
VvGA2ox7A y = -3.2219x + 39.64 R² = 0.9981 E = 103.98%
VvGA2ox7B y = -3.2782x + 37.211 R² = 0.9941 E = 98.197%
VvGA2ox7C y = -3.3029x + 35.993 R² = 0.9924 E = 100.804%
VvGA2ox8 y = -3.2803x + 32.785 R² = 0.9993 E = 101.771%
VvGAMT2 y = -3.3195x + 34.695 R2 = 0.9984 E = 100.101%
VvCYP714A1 y = -3.3251x + 30.695 R² = 0.9962 E = 99.864%
Ct value
Log10 cDNA dilution
Log10 cDNA dilution

Fig. 1-1. qRT-PCR efficiency plots for GA metabolic genes. Mean quantification cycle (C_T) values obtained from 10-fold serial dilution series of each gene plotted against the logarithm of cDNA template concentration. The amplification efficiency (E) was calculated by $E = [10^{(-1/S)} - 1] \times 100$, where S was the slope of the linear regression line.

RESULTS

Morphological changes in inflorescence in response to GA application

Grapevine inflorescence clusters were applied with or without GA at 14 DBF. Individual flowers were collected from the center of the clusters and the growth of the flowers was compared from 14 DBF to 5 days after full bloom (DAF). The flower was bigger with GA application than without GA application (Fig. 1-2A). With GA application, bigger ovary and ovule were observed in the flower at full bloom (Figs. 1-2B, C), indicating that GA stimulated the ovary and ovule development. Without GA application, pollen tubes grew normally after artificial pollination (Fig. 1-2D), but pollen tubes were coiled and swollen with GA application (Fig. 1-2E). Without GA application, seeds were normally developed (Fig. 1-2F), but seedlessness was induced with GA application at 14 DBF (Fig. 1-2G).

Identification of *Vitis* GA metabolic genes

Using amino acid sequences of *Arabidopsis* GA metabolic enzymes, five VvGA20ox, three VvGA3ox, and nine VvGA2ox family proteins, and one VvGAMT and one VvCYP714A proteins were identified (Tables 1-1, 1-2). The amino acid sequence comparisons revealed that *Vitis* GAoxs had a conserved Fe^{2+} 2-ODD domain in each ODD (Supplementary Figs. 1-1, 2, 3, 4, 5), as did GAoxs of *Arabidopsis* and rice. GA20oxs have two

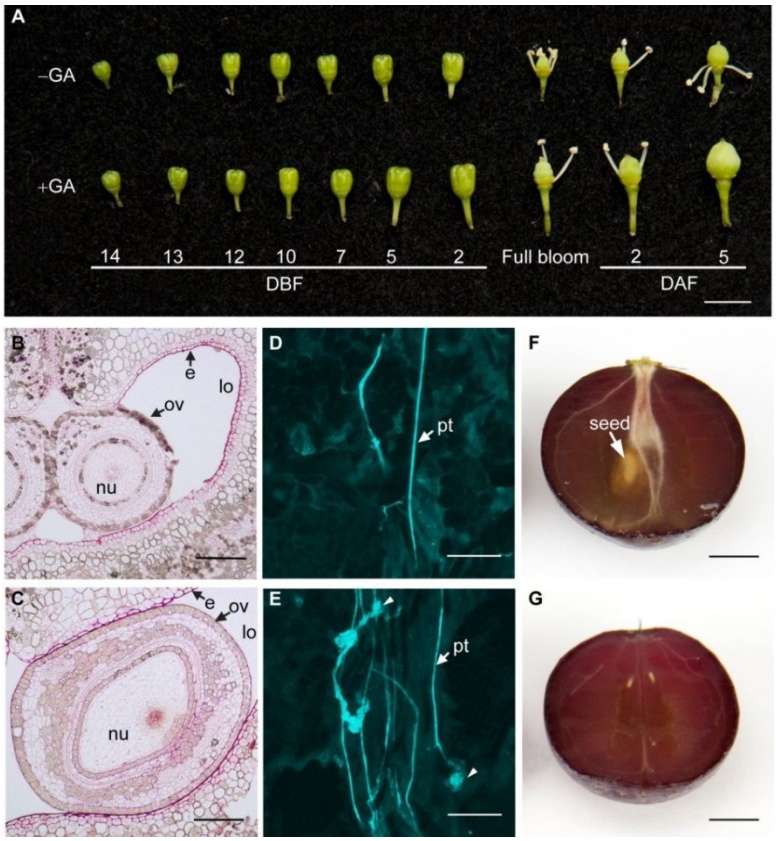

Fig. 1-2. Effects of GA application on grapevine inflorescence development. (A) comparison of the morphology of grapevine flowers without and with GA application from 14 DBF to 5 DAF. Scale bar is 0.5 cm. DBF, days before full bloom; DAF, days after full bloom. The ovaries at full bloom (B) without and (C) with GA application at 14 DBF were sectioned transversely. Scale bars are 100 μm. e, endodermis; lo, locule; nu, nucellus; ov, ovule. The pollen tube growth in the pistil (D) without and (E) with GA application was observed after 48 h artificial pollination, at 2 DAF. The artificial pollination was performed at full bloom using grapevine inflorescences emasculated at 1 DBF. Scale bars are 50 μm. Arrow heads indicate swollen and coiled pollen tubes. (F) Seeded and (G) seedless grape berries without and with GA application, respectively. Scale bars are 0.5 cm.

conserved sequences: LPWKET for GA substrate binding and NYYPXCXXP for 2-oxoglutarate binding site (Sakamoto et al., 2004; Song et al., 2011). The LPWKET sequence was conserved in VvGA20ox3, VvGA20ox4, and VvGA20ox5. VvGA20ox1 and VvGA20ox2 showed 61 and 56% identity to AtGA20ox1 and AtGA20ox2, respectively, but neither VvGA20ox1 nor VvGA20ox2 possessed the exact sequence (Supplementary Fig. 1-1). In the case of the 2-oxoglutarate binding site, NYYPXCXXP was conserved in all VvGA20ox family members. VvGA3oxs and VvGA2oxs also had conserved Fe^{2+} 2-ODD domains, but the homology of VvGA3ox family proteins was higher than that of the VvGA2ox family members. From VvGA3ox1 (55% identity to AtGA3ox1) to VvGA3ox3 (42% identity to AtGA3ox3), VvGA3oxs showed higher than 48% amino acid identity among VvGA3ox family members (Supplementary Fig. 1-2). In contrast, the AtGA2ox7 and AtGA2ox8 homologous *Vitis* proteins, VvGA2ox7A, VvGA2ox7B, VvGA2ox7C, and VvGA2ox8, showed lower than 23% identity to other VvGA2ox family proteins. In the case of VvGA2ox7C, some of the Fe^{2+} binding residues and 2-oxoglutarate binding residues were absent in its protein sequence (Supplementary Fig. 1-3). VvGAMT2 and VvCYP714A1 shared 59 and 57% identity with AtGAMT2 and AtCYP714A1, respectively. VvGAMT2 contained the S-adenosyl-L-methionine-dependent methyltransferase domain, as did AtGAMT2, and VvCYP714A1 had conserved heme binding motifs that are present in all cytochrome P450 proteins (Supplementary Figs. 1-4, 5).

Comparative nucleotide sequence analysis indicated that most of the *Vitis* GA metabolic genes shared more than 44% identity with the corresponding *Arabidopsis* genes (Supplementary Figs. 1-6, 7, 8, 9, 10). Based on these multiple alignment analyses, constructed phylogenetic trees showed that grapevines possessed a similar number of GA biosynthesis enzymes to *Arabidopsis* (Fig. 1-3). Unlike *Arabidopsis* GA catabolic enzymes, however, they had more subfamily members in GA2ox7, and only one GAMT2 and one CYP714A1 homologous proteins.

GA-induced transcriptional changes in *Vitis* GA biosynthetic genes

Seedlessness in grapevines could be induced when GA is appropriately applied at pre-bloom (Dass and Randhawa, 1968; Lu et al., 1997; Okamoto and Miura, 2005). Seedlessness was also induced in 'Tamnara' grapevines with GA application to their inflorescence at 14 DBF (Figs. 1-2F, G). After GA application, the relative expressions of *Vitis* GA metabolic genes were analyzed during inflorescence development. Transcriptional analysis revealed that expressions of *VvGA20oxs* were relatively even from 13 to 7 DBF and from 2 DBF to 5 DAF in the controls, except for *VvGA20ox3*. *VvGA20ox3* at 5 DBF was expressed at least 10-fold higher than the other *VvGA20ox* genes at the same stage in the controls (Fig. 1-4). In the GA3ox family, the highest *VvGA3ox2* expression level at 2 DBF exceeded the expression levels of the other *VvGA3ox* genes by more than 10-fold (Fig. 1-5). *VvGA3ox2* also showed the

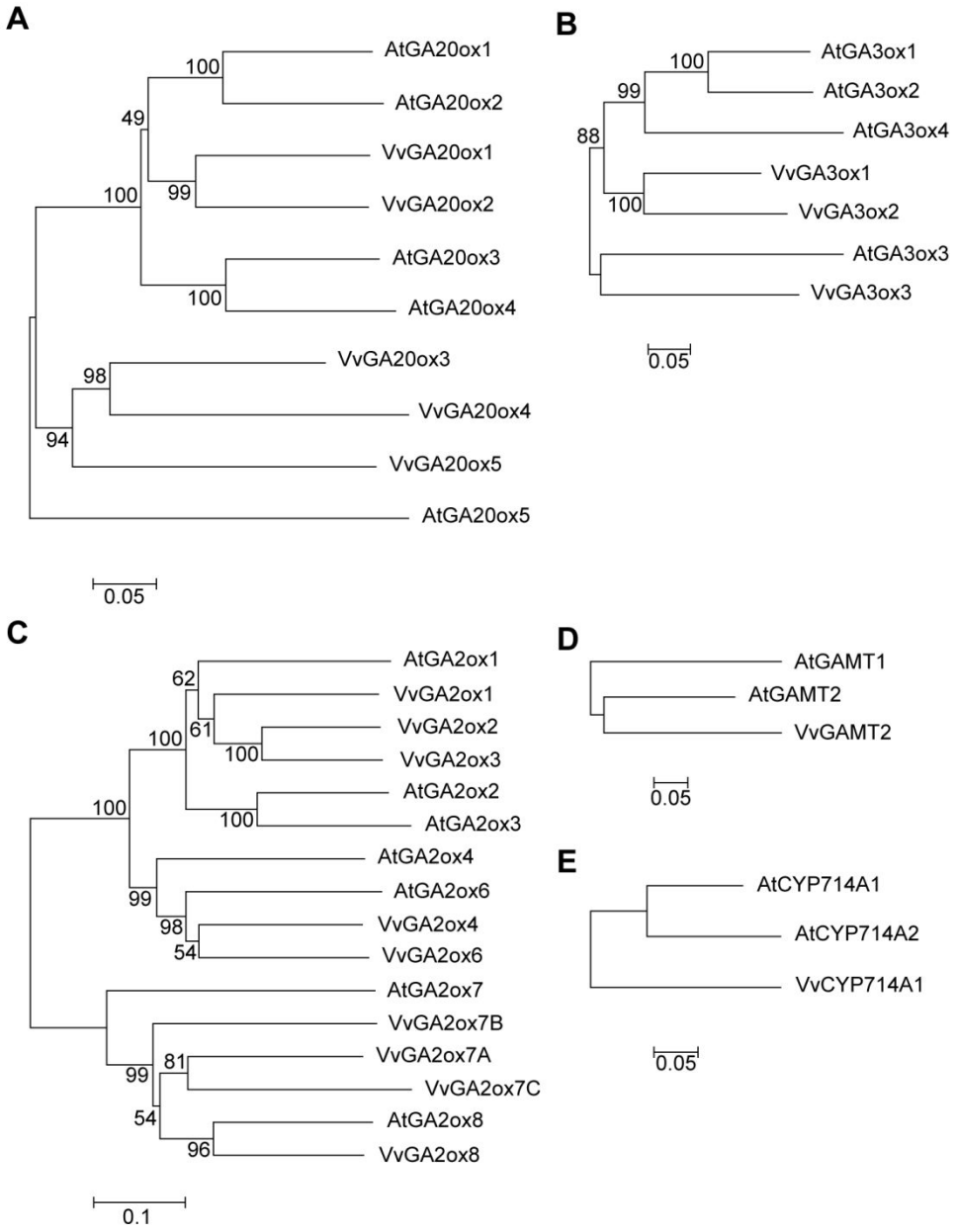

Fig. 1-3. Phylogenetic trees of *Vitis* and *Arabidopsis* GA metabolic enzymes. (A) VvGA20ox family. (B) VvGA3ox family. (C) VvGA2ox family. (D) VvGAMT2. (E) VvCYP714A1. At, *Arabidopsis thaliana*; Vv, *Vitis vinifera*.

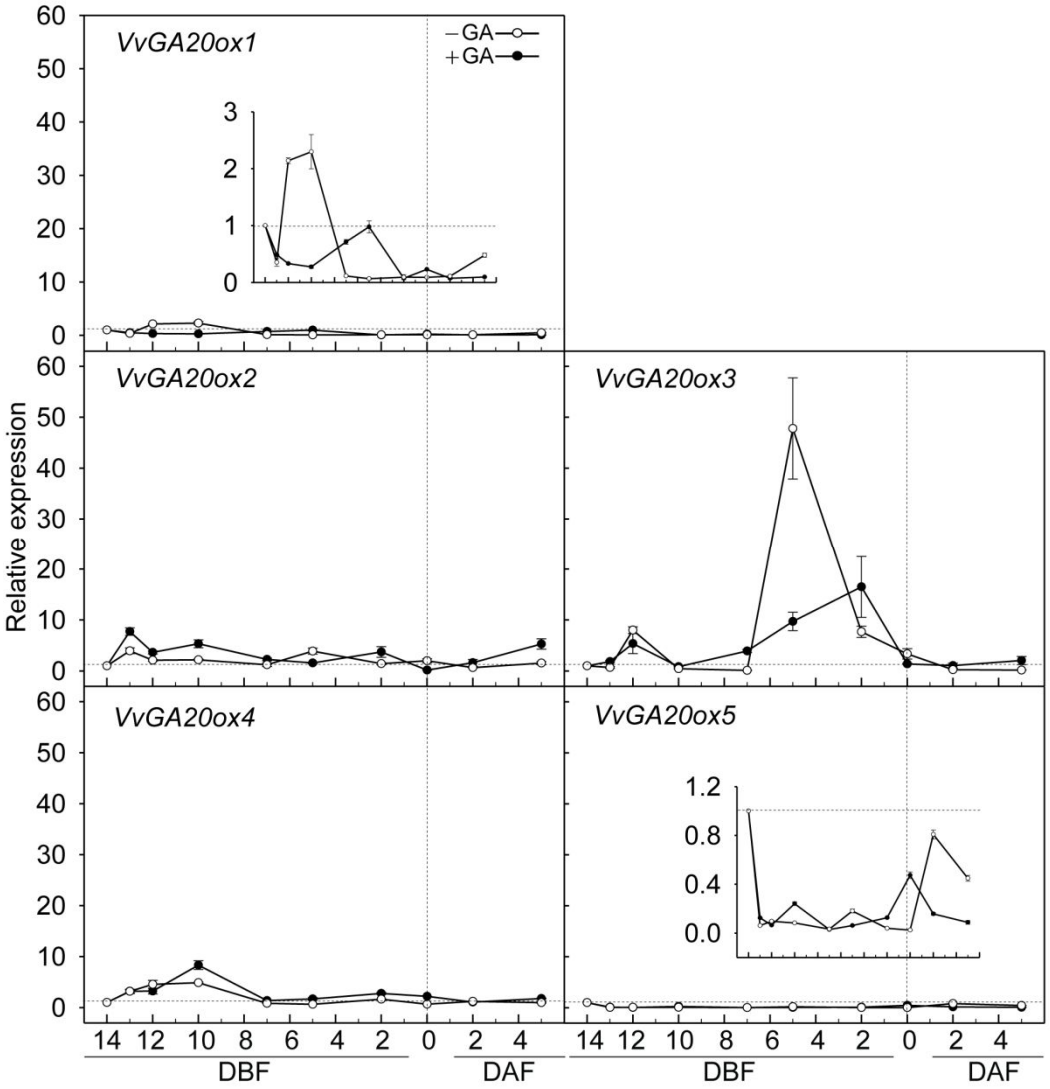

Fig. 1-4. Expression profiles of *VvGA20oxs* in inflorescence tissues without and with GA application. The expression levels of *VvGA20ox1* and *VvGA20ox5* are magnified in the insets. Error bars represent standard errors from three independent PCR amplifications and quantifications. DBF, days before full bloom; 0, full bloom; DAF, days after full bloom.

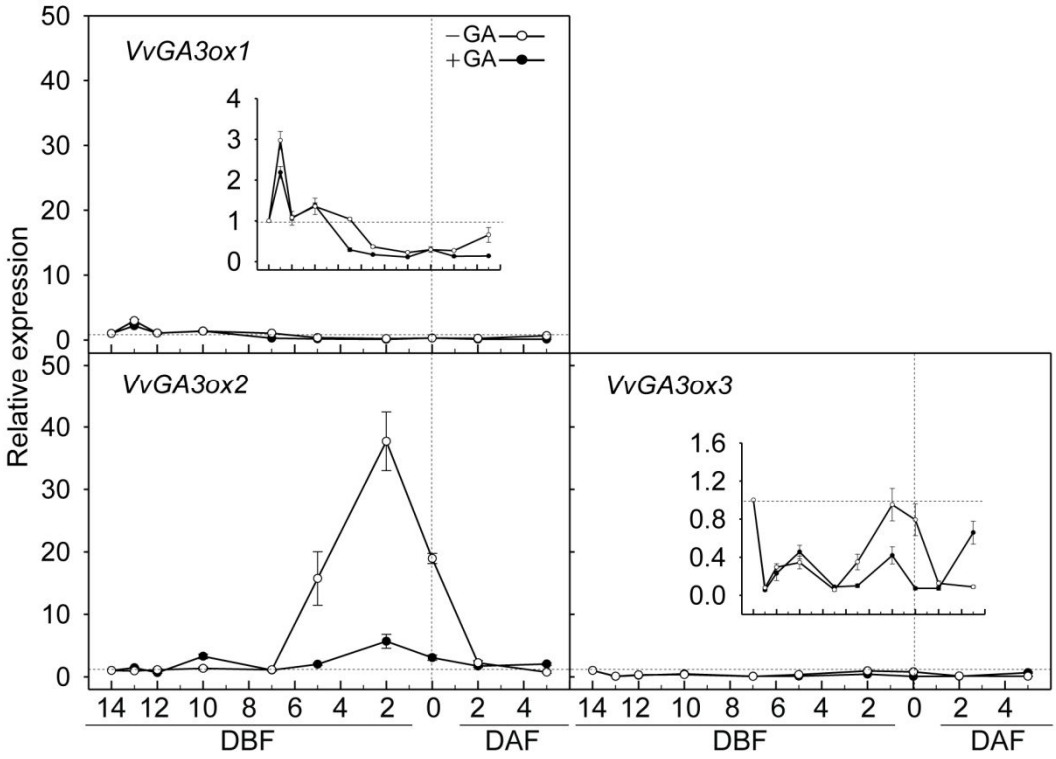

Fig. 1-5. Expression profiles of *VvGA3oxs* in inflorescence tissues without and with GA application. The expression levels of *VvGA3ox1* and *VvGA3ox3* are magnified in the insets. Error bars represent standard errors from three independent PCR amplifications and quantifications. DBF, days before full bloom; 0, full bloom; DAF, days after full bloom.

increased expression patterns at 5 DBF to full bloom. These expression patterns suggest that grapevines mainly use *VvGA20ox3* and *VvGA3ox2* in GA biosynthesis during inflorescence development.

After GA application, *VvGA20ox2* and *VvGA20ox4* were up-regulated depending on the developmental stages, but most of the *VvGA20ox* and *VvGA3ox* family genes were down-regulated throughout the inflorescence development (Figs. 1-4, 1-5). In the cases of *VvGA20ox1*, *VvGA20ox3*, and *VvGA3ox2*, the transcription levels upon GA application were reduced over 70% at 10, 5, and 2 DBF, respectively. The peak expression timing of *VvGA20ox3* was also changed with GA application. In the controls, the expression levels of *VvGA20ox3* peaked at 5 DBF, however, *VvGA20ox3* expression was highest at 2 DBF with GA application.

GA-induced transcriptional changes in *Vitis* GA catabolic genes

GA2ox, GAMT, and CYP714A, catabolic regulators for GA levels, were encoded by multiple genes in higher plants with differential expressions (Hedden and Phillips, 2000; Schomburg et al., 2003; Thomas et al., 1999; Varbanova et al., 2007; Zhang et al., 2011). The relative expressions of all *Vitis* GA catabolic genes identified in phylogenetic analysis were also analyzed in the control or GA-applied grapevine inflorescence tissues. The overall transcription levels of the *VvGA2ox* gene family were low from 13 DBF to 5 DAF, with the exceptions of *VvGA2ox1*, *VvGA2ox3*, and *VvGA2ox7A* (Fig. 1-6). In controls, *VvGA2ox1* was highly expressed from

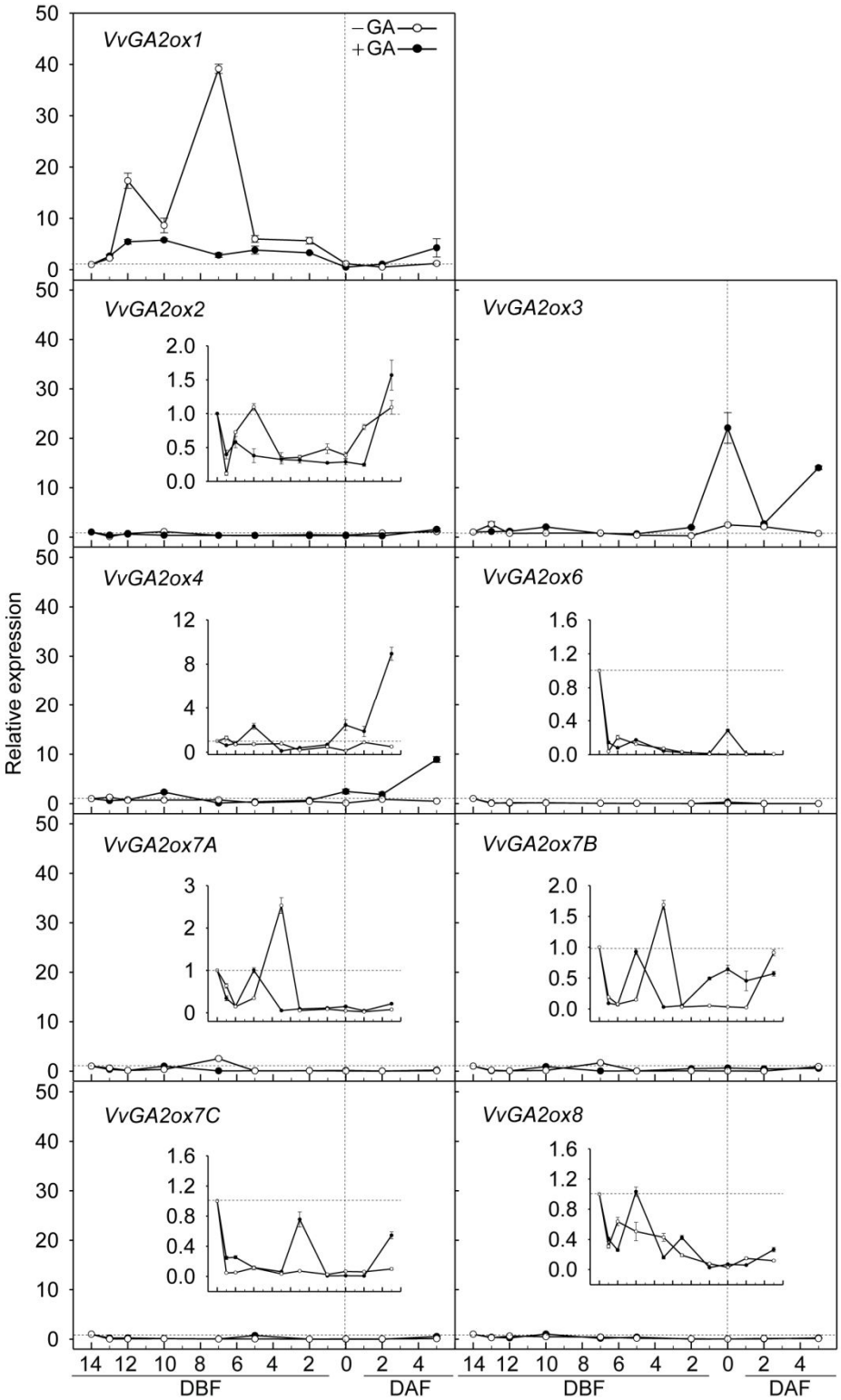
VvGA2ox1
−GA
+GA
VvGA2ox2
VvGA2ox3
VvGA2ox4
VvGA2ox6
VvGA2ox7A
VvGA2ox7B
VvGA2ox7C
VvGA2ox8
Relative expression
14 12 10 8 6 4 2 0 2 4
DBF
DAF

Fig. 1-6. Expression profiles of *VvGA2oxs* in inflorescence tissues without and with GA application. The expression levels of *VvGA2oxs*, except *VvGA2ox1* and *VvGA2ox3*, are magnified in the insets. Error bars represent standard errors from three independent PCR amplifications and quantifications. DBF, days before full bloom; 0, full bloom; DAF, days after full bloom.

12 to 2 DBF, and both *VvGA2ox3* and *VvGA2ox7A* also showed higher expression patterns than other *VvGA2oxs*, depending on the developmental stage. However, the peak expression of *VvGA2ox1* at 7 DBF was almost 20-fold higher than the expressions of other *VvGA2ox* gene family members. These results imply that *VvGA2ox1* is the major GA catabolic gene among *VvGA2oxs* in grapevine inflorescence. Another GA catabolic gene, *VvGAMT2* showed gradually reduced expression patterns in the controls during later developmental stages (Fig. 1-7), while expression levels of *VvCYP714A1* increased until 2 DBF and subsequently decreased (Fig. 1-8).

Of the GA catabolic genes, variations in expression were observed for individual *VvGA2ox* genes upon GA application (Fig. 1-6). *VvGA2ox1*, *VvGA2ox7A*, and *VvGA2ox7B* were down-regulated over 90% at 7 DBF after GA application. In contrast, the transcriptional level of *VvGA2ox3* was increased by over 9-fold at full bloom and 19-fold at 5 DAF, and *VvGA2ox4* was up-regulated by almost 17-fold at 5 DAF, compared to the controls after GA application. *VvGAMT2* showed GA-dependent increases in transcription of 9-fold at 12 DBF and 7-fold at 10 DBF (Fig. 1-7). Moreover, *VvGAMT2* showed the earliest and highest transcription level after GA application, and this GA-mediated up-regulation of *GAMT2* has not yet been reported. With GA application, transcription levels of *VvCYP714A1* also increased more than 3-fold at 12 DBF and 1.5-fold at 2 DBF (Fig. 1-8).

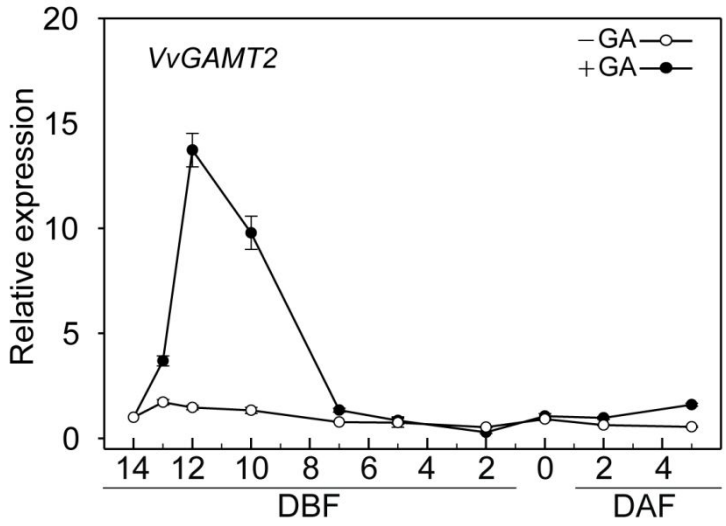

Fig. 1-7. Expression profiles of *VvGAMT2* in inflorescence tissues without and with GA application. Error bars represent standard errors from three independent PCR amplifications and quantifications. DBF, days before full bloom; 0, full bloom; DAF, days after full bloom.

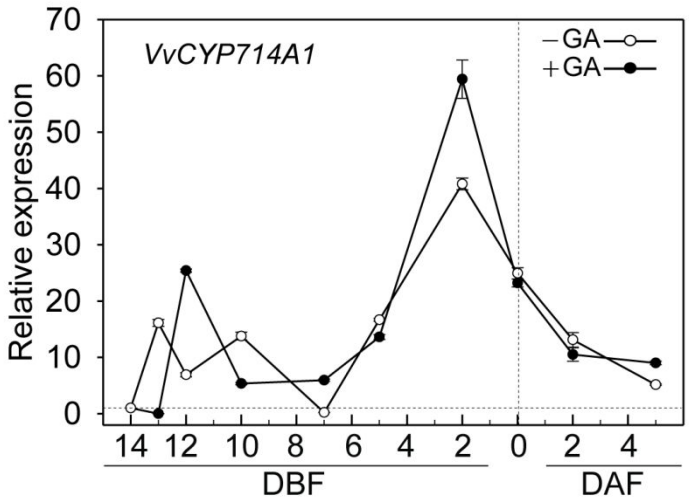

Fig. 1-8. Expression profiles of *VvCYP714A1* in inflorescence tissues without and with GA application. Error bars represent standard errors from three independent PCR amplifications and quantifications. DBF, days before full bloom; 0, full bloom; DAF, days after full bloom.

DISCUSSION

Characterization of *Vitis* GA metabolic genes

Since the first report of *AtGA20ox1* by Phillips et al. (1995), GAoxs, critical determinants for bioactive GA levels, have been characterized in various plant species. Among five AtGA20oxs, all rice and maize GA20oxs were AtGA20ox5 closely related homologies (Plackett et al., 2012; Sakamoto et al., 2004; Song et al., 2011). However, SlGA20oxs in tomato showed higher similarity with AtGA20ox1 and AtGA20ox2 than with AtGA20ox5 (Serrani et al., 2007). The phylogenetic analysis and amino acid sequence alignment in this study showed that grapevines also had multigene-encoded GA20oxs with two clades (Fig. 1-3A, Supplementary Fig. 1-1). One clade includes AtGA20ox1 and AtGA20ox2, closely related to VvGA20ox1 and VvGA20ox2, which are missing an LPWKET residue, and the other clade includes AtGA20ox5-related to VvGA20ox3, VvGA20ox4, and VvGA20ox5. Except in the cases of VvGA20ox1 and VvGA20ox2, VvGA20ox family proteins share two conserved sequences, an LPWKET residue for GA substrate binding and NYYPXCXXP for the 2-oxoglutarate binding site. All three VvGA3oxs and nine VvGA2oxs also possess an Fe^{2+} 2-ODD domain with different homologies within each protein family. Both VvGA3ox1 and VvGA3ox2 showed higher homology with AtGA3ox4 (Fig. 1-3B), as did SlGA3ox1 and SlGA3ox2, but close homology of VvGA3ox3 with AtGA3ox3, as seen in rice and maize

GA3oxs, was also observed. Unlike tomato SlGA2oxs (Serrani et al., 2007), VvGA2oxs possessed C_{20}-GA substrate-favoring AtGA2ox7 and AtGA2ox8 homologous members, VvGA2ox7A, VvGA2ox7B, VvGA2ox7C, and VvGA2ox8 (Fig. 1-3C), more than did *Arabidopsis* (Schomburg et al., 2003), rice (Sakamoto et al., 2004), and maize (Song et al., 2011).

VvGA2ox1, _VvGA20ox3_, and _VvGA3ox2_ are sequentially expressed during inflorescence development without GA application

GA metabolic genes are differentially expressed according to developmental stage in *Arabidopsis* (Plackett et al., 2012; Rieu et al., 2008; Thomas et al., 1999; Varbanova et al., 2007; Zhang et al., 2011), rice (Sakamoto et al., 2004; Zhu et al., 2006), and tomato (Serrani et al., 2007). The expression levels of *GAoxs* have been reported to correlate with GA content during inflorescence development (Ozga et al., 2003, 2009; Rieu et al., 2008; Serrani et al., 2007). For example, *GA2oxs* are up-regulated before pollination, but down-regulated after pollination. However, *GA20oxs* and *GA3oxs* are up-regulated after pollination. In the present study, *VvGA2ox1* was highly expressed at 7 DBF, and the peak expressions of *VvGA20ox3* and *VvGA3ox2* were observed at near full bloom (Figs. 1-4, 1-5, 1-6), showing sequential expression of these genes. These results might account for the elevated GA levels upon pollination in grapevines (Wang et al., 1993). In addition, *VvGAMT2* was expressed insignificantly (Fig. 1-7), but the expression of *VvCYP714A1* gradually

increased until 2 DBF (Fig. 1-8). The expression pattern of *VvCYP714A1* is similar to that of *Elongated uppermost internode* (*EUI*) encoding 16α, 17-epoxidase in rice, which is highly expressed in the flower even at the heading stage, anthesis (Zhu et al., 2006), although the exact roles of CYP714A1 and CYP714A2 in GA metabolism remain unclear.

GA application at pre-bloom alters transcription levels of *Vitis* GA metabolic genes at near full bloom

Bioactive GA level influences transcription of GA metabolic genes. With GA application, down-regulation of GA biosynthetic genes and up-regulation of GA catabolic genes have been observed in *Arabidopsis* (Rieu et al., 2008; Thomas et al., 1999) and tobacco (Gallego-Giraldo et al., 2008). Following GA application, two highly expressed GA biosynthesis genes, *VvGA20ox3* and *VvGA3ox2*, were greatly down-regulated at 5 DBF and from 5 DBF to full bloom, respectively (Figs. 1-4, 1-5). However, GA catabolic genes, such as *VvGA2ox* gene family, showed more complex transcriptional changes after GA application. *VvGA2ox3* and *VvGA2ox4* were up-regulated at full bloom and 5 DAF, respectively, and *VvGA2ox1*, the most highly expressed gene among *VvGA2oxs*, was down-regulated. With GA application, *VvCYP714A1* was up-regulated slightly only at 12 and 2 DBF, but *VvGAMT2* was rapidly up-regulated by more than 7-fold from 12 to 10 DBF (Figs. 1-7, 1-8); this is the first report of up-regulation of *GAMTs* by exogenous GA.

The peak expression timing changes were compared in the most highly expressed genes in each *Vitis* GA metabolic gene family without GA application and up-regulated genes over 9-fold with GA application. *VvGA2ox1*, *VvGA20ox3*, and *VvGA3ox2* were sequentially expressed at 7, 5, and 2 DBF, respectively (Fig. 1-9A). GA application down-regulated *VvGA20ox3* and *VvGA3ox2* and up-regulated GA catabolic genes at near full bloom (Fig. 1-9B). These observations indicate that GA application to grapevine inflorescence at pre-bloom alters the transcription levels of GA metabolic genes towards a decrease in bioactive GA level.

GA application and seedlessness

GA application to grapevine inflorescence at pre-bloom increased ovary and ovule size (Figs. 1-2A, B, C), arrested pollen tube growth (Figs. 1-2D, E), and induced parthenocarpic fruit development (Figs. 1-2F, G). Pollen tube growth inhibition induced by GA application was also reported in 'Delaware' grapevine (Okamoto and Miura, 2005), without causing any defects in the pistil. For example, cell layer number of transmitting tissues in the pistil was not affected by GA application (Okamoto and Miura, 2005). Similarly, pollen tube growth was inhibited in *Arabidopsis* mutants overexpressing the GA catabolic gene *GA2ox2* (Singh et al., 2002) and in mutants lacking the GA biosynthesis genes *ga3ox1* and *ga3ox3* (Hu et al., 2008), consistent with the reduction of *VvGA20ox3* and *VvGA3ox2* at near full bloom with GA application in the present study (Figs. 1-4, 1-5). With

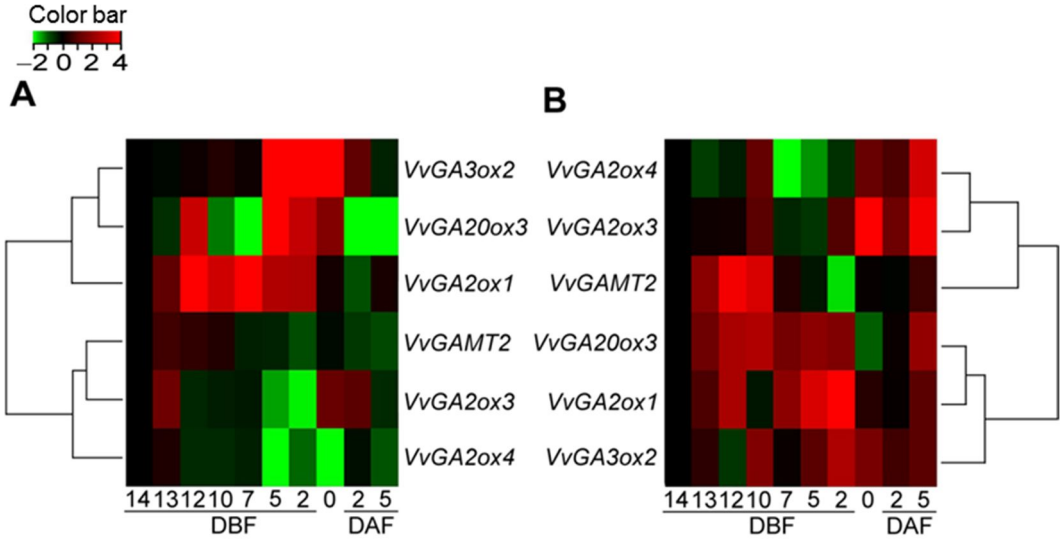

Fig. 1-9. Expression profiles of *Vitis* GA metabolic genes *VvGA20ox3*, *VvGA3ox2*, *VvGA2ox1*, *VvGA2ox3*, *VvGA2ox4*, and *VvGAMT2* during inflorescence development. (A) Relative expression of each gene without GA application. (B) Relative expression of each gene after GA application was compared depending on the inflorescence developmental stage. Color bar represents $\log_2$ expression values, increased and decreased gene expressions are colored red and green, respectively. DBF, days before full bloom; 0, full bloom; DAF, days after full bloom.

GA application, therefore, fertilization could not occur due to the inhibited pollen tubes, but ovaries enlarged and developed into parthenocarpic fruit.

Enlarged ovary induced by GA application has been reported in tomato, and it shows increase of cell size in the internal mesocarp (Serrani et al., 2007). The *parthenocarpic fruit* (*pat*) mutant overexpressing GA20ox in tomato exhibited enlarged ovaries, but with aberrant development of anther and ovule (Mazzucato et al., 1998; Olimpieri et al., 2007). These results imply that GA triggers metabolic changes inducing parthenocarpic fruit development. This implication could be substantiated by the observations that hormone balance was disrupted by GA application when transmitting tissues were initiated to develop in the style (Okamoto et al., 2002) and expressions of GA oxidase genes were altered in the mutants overexpressing *GA2ox2* gene (Singh et al., 2002).

Parthenocarpic fruit development in tomato could also be induced by auxin application or silencing of auxin-related genes, such as *SlARF7* or *SlIAA9* (de Jong et al., 2009b; 2011; Wang et al., 2005). Although fruit cell expansion is normally regulated by increased GA level after fertilization, auxins have the effects on GA signaling with auxin-related genes and on the GA level itself to initiate cell expansion (de Jong et al., 2009, 2011). Similarly to the effects of GA application in the present study, pollen tube growth inhibition (de Jong et al., 2009b) and alteration in expressions of GA oxidase genes and GA level (de Jong et al., 2011) have been observed in *ARF7* silencing mutants. Altered expressions of auxin

signaling genes were reported in mutants overexpressing *SlDELLA*, a GA signaling gene (Carrera et al., 2012). Since GA application changes not only the GA levels but also the interaction of GA with other hormones including auxins, however, the characterization of *Vitis* GA metabolic gene expression might not be enough to account for how parthenocarpic fruit development could be induced in seeded grapevine cultivars upon GA application. The influences of GA application on auxin level and auxin-related gene expression should be investigated to further understand GA-induced seedlessness in grapevines.

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CHAPTER 2

Gibberellin Application at Pre-bloom in Grapevines Down-regulates the Expressions of *VvIAA9* and *VvARF7*, Negative Regulators of Fruit Set Initiation, during Parthenocarpic Fruit Development

ABSTRACT

Fruit set is initiated only after fertilization and is tightly regulated primarily by gibberellins (GAs) and auxins. The application of either of these hormones induces parthenocarpy, fruit set without fertilization, but the molecular mechanism underlying this induction is poorly understood. In the present study, the parthenocarpic fruits induced by GA application at pre-bloom were found to result from the interaction of GA with auxin signaling. The transcriptional levels of the putative negative regulators of fruit set initiation, including *Vitis auxin/indole-3-acetic acid transcription factor 9* (*VvIAA9*), *Vitis auxin response factor 7* (*VvARF7*), and *VvARF8* were monitored during inflorescence development in seeded diploid 'Tamnara' grapevines with or without GA application. Without GA application, *VvIAA9, VvARF7*, and *VvARF8* were expressed at a relatively high level before full bloom, but decreased thereafter following pollination. After GA application at 14 days before full bloom (DBF); however, the

expression levels of *VvIAA9* and *VvARF7* declined at 5 DBF prior to pollination. The effects of GA application on auxin levels or auxin signaling were also analyzed by monitoring the expression patterns of auxin biosynthesis genes and auxin-responsive genes with or without GA application. Transcription levels of the auxin biosynthesis genes *Vitis anthranilate synthase β subunit* (*VvASB1-like*), *Vitis YUCCA2* (*VvYUC2*), and *VvYUC6* were not significantly changed by GA application. However, the expressions of *Vitis Gretchen Hagen3.2* (*VvGH3.2*) and *VvGH3.3*, auxin-responsive genes, were up-regulated from 2 DBF to full bloom with GA application. Furthermore, the *Vitis* GA signaling gene, *VvDELLA* was up-regulated by GA application during 12 DBF to 7 DBF, prior to down-regulation of *VvIAA9* and *VvARF7*. These results suggest that *VvIAA9* and *VvARF7* are negative regulators of fruit set initiation in grapevines, and GA signaling is integrated with auxin signaling via *VvDELLA* during parthenocarpic fruit development in grapevines.

INTRODUCTION

Fruit set is initiated only after two sequential events, pollination and fertilization (Gillaspy et al., 1993), concurrent with changes in the levels of endogenous plant hormones, primarily gibberellins (GAs) and auxins (Coombe, 1960; Kuhn and Arce-Johnson, 2012; Ozga and Reinecke, 2003; Wang et al., 1993). Application of GA or auxin can trigger fruit set even without pollination and can induce parthenocarpic fruit development (de Jong et al., 2009a; Gustafson, 1936; Schwabe and Mills, 1981; Vivian-Smith and Koltunow, 1999; Wittwer et al., 1957).

Although the molecular mechanisms by which either GA or auxin mediates fruit set initiation are not clearly established, several auxin signaling genes related to parthenocarpic fruit development have been identified. The auxin/indole-3-acetic acid (Aux/IAA) transcription factor family is a known essential repressor of auxin signaling in various developmental processes, including fruit set (Ulmasov et al., 1997). Among the Aux/IAA family genes, *IAA9* has been regarded as a negative regulator, preventing fruit set initiation in the absence of pollination in tomato, with the silencing line, *SlIAA9*, showing parthenocarpic fruit development (Wang et al., 2005). Two auxin response factors (ARF) related to parthenocarpic fruit development have also been identified in *Arabidopsis* and tomato (de Jong et al., 2009b; Goetz et al., 2006; 2007; Wang et al., 2005). The ARF family acts as a regulator of auxin-responsive genes by specifically binding to auxin response elements (AuxREs) in the promoters of auxin-responsive genes. ARFs play important roles in diverse

developmental processes in embryos, hypocotyls, floral organs, and fruit (Finet et al., 2006; Hardtke et al., 1998; Harper et al., 2000; Li et al., 2004; Li et al., 2006; Schruff et al., 2006; Wang et al., 2005). The *Arabidopsis fruit without fertilization* (*fwf*) mutant produces parthenocarpic fruit as the result of the expression of truncated *ARF8* (Goetz et al., 2006; 2007), and the same mutation in the *SlARF8* in tomato induced parthenocarpy (Goetz et al., 2007). Parthenocarpy induction and pollen tube growth inhibition were also observed in a silencing line of *ARF7* in tomato (de Jong et al., 2009b; 2011). The expression of *SlARF7* was maintained at high levels before pollination and rapidly declined after pollination with increasing auxin content (Coombe, 1960; de Jong et al., 2009a; Gillaspy et al., 1993; Kuhn and Arce-Johnson, 2012), in a manner similar to its down-regulation upon exogenous auxin application (de Jong et al., 2009b).

GA-induced parthenocarpic fruit development has been observed in tomato mutants (*pat*, *pat-2*, and *pat-3/pat*-4) showing overexpression of the GA biosynthesis genes (Fos et al., 2000; 2001; Olimpieri et al., 2007), and in silencing lines of *DELLA* in *Arabidopsis* (Fuentes et al., 2012) and tomato (Marti et al., 2007), demonstrating that GA signaling plays a role in parthenocarpic fruit development. The regulatory roles of the DELLA protein in *ARF7* expression, and the partial activation of auxin signaling during parthenocarpic fruit development have been reported in the *procera* (*pro*) mutant of *DELLA* in tomato (Carrera et al., 2012). Additionally, auxin application and the silencing line of *ARF7* induced parthenocarpy by regulating expression levels of the GA metabolic genes (de Jong et al., 2011; Dorcey et al., 2009; Serrani et al., 2008). These data suggest that

both GA and auxin influence fruit set initiation, and crosstalk between GA and auxin signaling plays a role in parthenocarpic fruit development.

In grapevines, GA has commonly been used to induce parthenocarpy (Coombe, 1960; Gustafson, 1936; Lu et al., 1997; Schwabe and Mills, 1981). The effects of GA application have been studied on early ripening and berry enlargement (Casanova et al., 2009; Dokoozlian et al., 2001; Iwahori et al., 1968), and on the appropriate application timing at the pre-bloom stage for the induction of seedless grapes (Lu et al., 1997; Okamoto and Miura, 2005). On a molecular level, both GA and auxin biosynthesis genes are up-regulated after pollination in grapevines (Dauelsberg et al., 2011; Jung et al., 2014; Kuhn and Arce-Johnson, 2012). However, although GA-mediated parthenocarpy is a highly desirable trait for table grapes, how GA induces parthenocarpic fruit development remains unclear. GA application at the pre-bloom stage in grapevines inhibited pollen tube growth and disturbed the balance of GA metabolism at near full bloom (Jung et al., 2014). In the present study, changes in the expression levels of the fruit set related genes, *VvIAA9*, *VvARF7*, *VvARF8*, and *VvDELLA* in grapevines were monitored to determine whether GA application coordinates auxin signaling during parthenocarpic fruit development.

MATERIALS AND METHODS

Plant material and GA application

Five-year-old grapevines of the seeded diploid cultivar ‘Tamnara’ (*Vitis* spp.), grown in an overhead arbor system, were used for the GA application and gene expression analysis. The cultivar used in this study was bred from a cross between ‘Campbell Early’ (*V. labruscana*) and ‘Himrod’ (*Vitis* spp.) at the National Institute of Horticultural and Herbal Science, Suwon, Republic of Korea in 1998 (Park et al., 2004). A GA solution (Dongbu, Seoul, Korea) at 100 ppm was applied as described by Okamoto and Miura (2005) onto inflorescence clusters 14 days before full bloom (DBF), which corresponded to the stage showing eight separated leaves and a compact grouped flower, based on the E-L system of Coombe (1995), and they were labeled. Clusters were harvested at 0, 1, 2, 4, 7, 9, 12, 14, 16, and 19 days after GA application. Harvested inflorescence samples were immediately frozen in liquid N_2, and stored at –80°C until RNA extraction.

Protein identification

The *Vitis* homologous proteins ARF7, ARF8, YUCCA2 (YUC2), YUC6, and DELLA were identified using a BLASTP search, except for the previously identified *Vitis* IAA9 (VvIAA9) (HQ337788) (Fujita et al., 2012) and VvGAI1 (XP_002284648) (Boss and Thomas, 2002; Zhong and Yang,

2012). Amino acid sequences for the *Arabidopsis* ARF, Aux/IAA, YUC, and DELLA family proteins were obtained from the National Center for Biotechnology Information (NCBI). For tomato, however, amino acid sequences of the homologues were acquired from previous genome-wide studies (Expósito-Rodríguez et al., 2011; Kumar et al., 2012; Wu et al., 2012). Using these sequences, iTAK (http://bioinfo.bti.cornell.edu/cgi-bin/itak/index.cgi), the Plant Transcription Factor Database, version 3.0 (PlnTFDB, http://plntfdb.bio.uni-potsdam.de/v3.0/), and GreenPhyl (http://www.greenphyl.org/cgi-bin/index.cgi) were screened, and protein sequences were confirmed using the NCBI. The gene sequences of *VvARF7* (GSVIVT01015035001), *VvARF8* (GSVIVT01035204001), *VvYUC2* (GSVIVT01015388001), *VvYUC6* (GSVIVT01035678001), and *VvDELLA* (GSVIVT01030735001) were identified using the Grape Genome Browser, version 12X (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). Distinct domains for each protein were predicted using PROSITE (http://prosite.expasy.org/prosite.html), Pfam (http://pfam.janelia.org/), and previous reports of ARFs (de Jong et al., 2009b; Kumar et al., 2011; Ulmasov et al., 1999), YUCs (Cheng et al., 2006; Kim et al., 2011), and DELLAs (Bassel et al., 2004). Accession numbers for all amino acid sequences used in this study are listed in Supplementary Table 2-1.

Phylogenetic analysis

Protein sequence alignments were generated using ClustalW version

2.1 for multiple alignments, and phylogenetic analyses were performed using neighbor-joining algorithms of the MEGA5 program (Tamura et al., 2011) with the pairwise-deletion option. One thousand replicates were used in the bootstrap analysis.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from whole inflorescence samples including berries and pedicels, at various developmental stages according to an RNA extraction method (Chang et al., 1993) modified to remove polysaccharides and phenolic compounds. The cDNA was synthesized by reverse transcription of 0.5 μg RNA using the PrimeScript first-strand cDNA Synthesis Kit (Takara, Tokyo, Japan) with an oligo-dT primer, according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

cDNA was subjected to qRT-PCR using the gene-specific forward and reverse primers shown in Supplementary Table 2-2. Primers used in previous studies (Böttcher et al., 2011; Dauelsberg et al., 2011) were used for the grapevine auxin biosynthesis gene, the putative *Vitis* anthranilate synthase β subunit-like (*VvASB1-like*), and two auxin signaling *Vitis Gretchen Hagen3* (*GH3*) family genes. qRT-PCR was performed with the SYBR Premix Ex Taq (Takara) on a Thermal Cycler Dice Real-Time

System TP800, version 4.0 (Takara), under universal thermal cycling conditions described by the manufacturer. *Vitis Actin1* was used as an endogenous control for normalization of gene expression. With cycle threshold (C_T) values obtained from the qRT-PCR results, the ΔC_T value ($C_{T\ \text{target gene}} - C_{T\ \textit{VvActin1}}$) was calculated for each gene. The relative expression of each gene normalized to the ΔC_T value of samples from 14 DBF was determined using the comparative C_T method ($2^{-\Delta\Delta CT}$). Analysis of qRT-PCR efficiency showed that all amplicons of all genes used in this study were in the optimal range of 95-105% (Fig. 2-1).

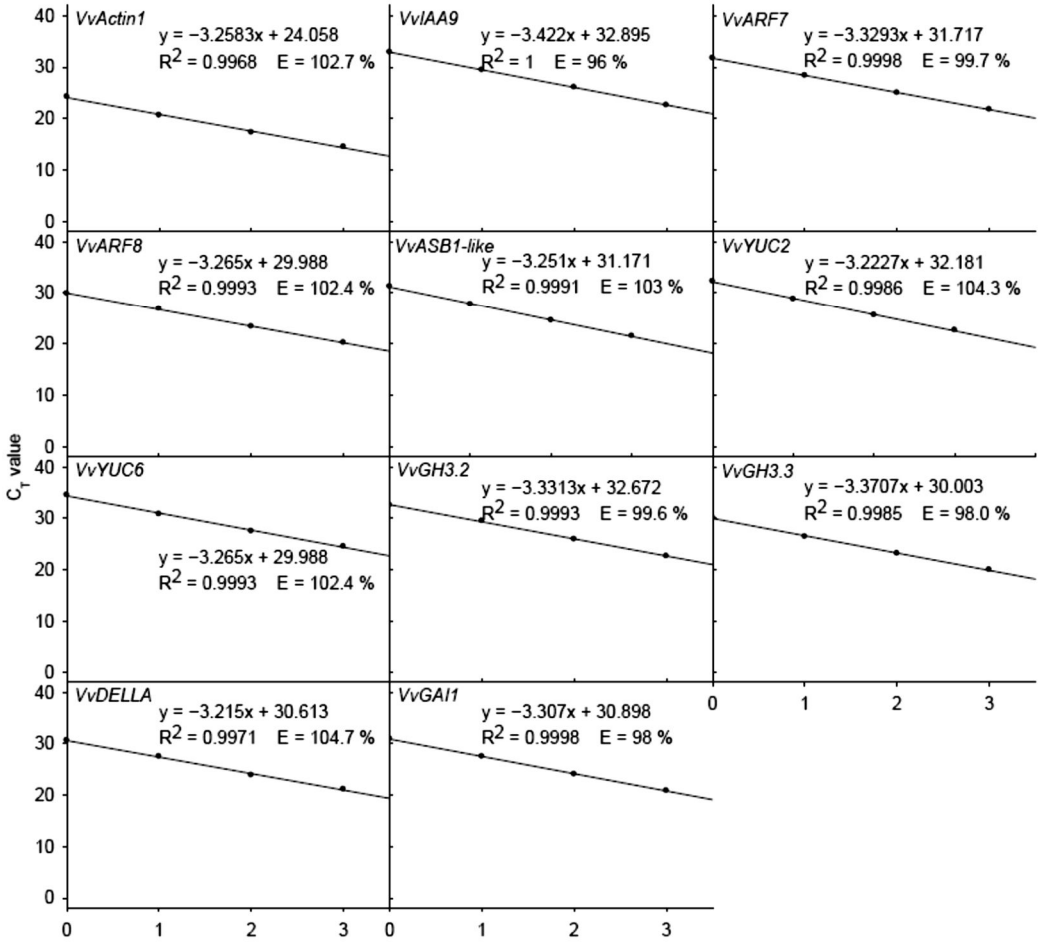


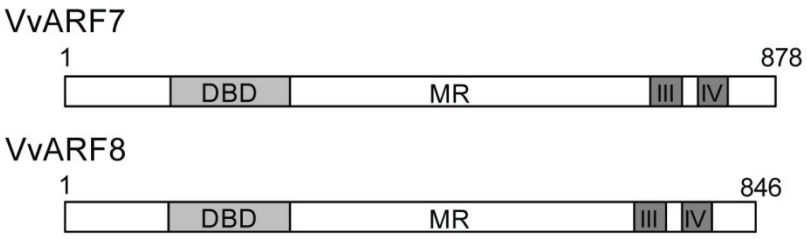
Fig. 2-1. qRT-PCR efficiency plots. Mean quantification cycle (C_T) values obtained from 10-fold serial dilution series of each gene plotted against the logarithm of cDNA template concentration. The amplification efficiency (E) was calculated by $E = [10^{(-1/S)} - 1] \times 100$, where S was the slope of the linear regression line.

RESULTS

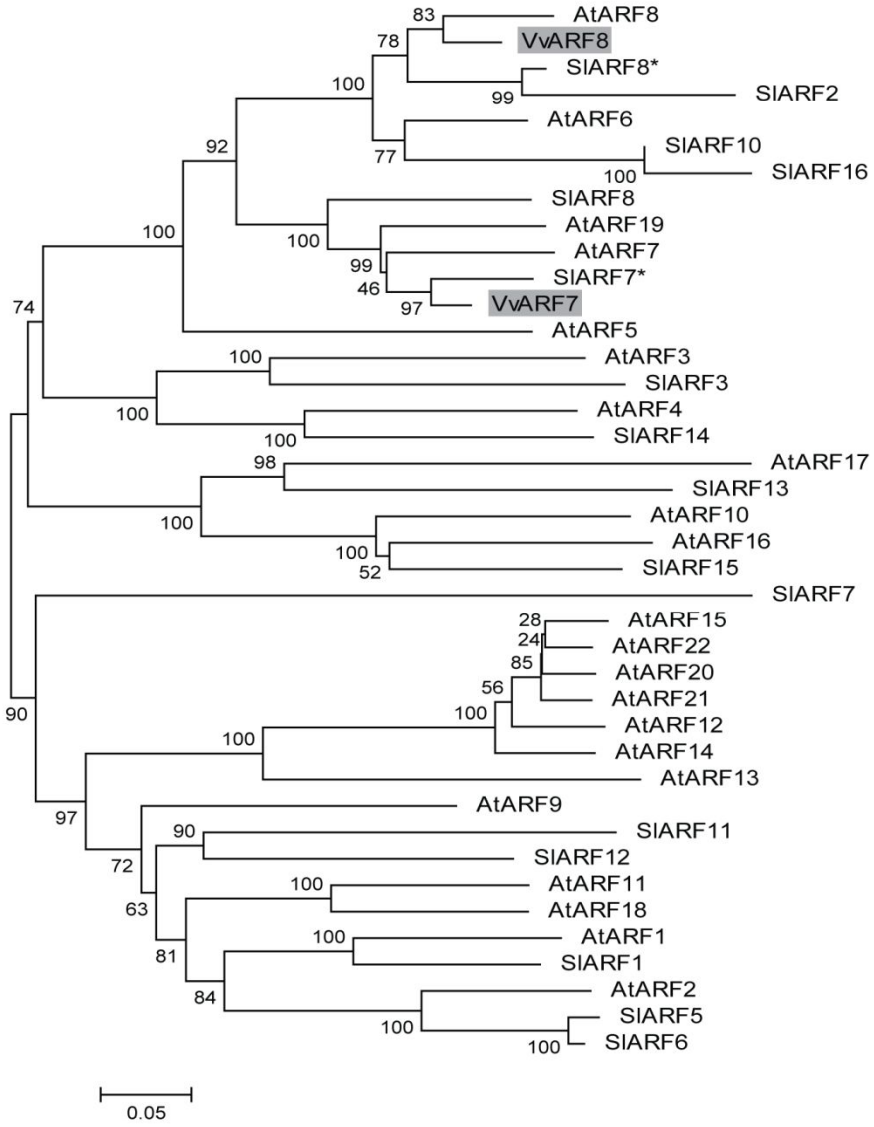
Characterization and identification of *Vitis* ARF7 and ARF8

Two ARF family proteins, VvARF7 and VvARF8, were identified from several *Vitis* transcription factor databases. VvARF7 and VvARF8 were previously reported as VvIAA24 and VvIAA7, respectively (Çakir et al., 2013). Using several plant transcription databases and PROSITE and Pfam, the VvARF7 amino acid sequence was deduced to contain an N-terminal B3-type DNA-binding domain (DBD; amino acids 126-228), a middle region (MR), two C-terminal Aux/IAA dimerization domains, domain III (amino acids 753-788), and domain IV (amino acids 797-839), needed for typical ARF protein activities. The VvARF8 sequence was also deduced to contain an N-terminal DBD (amino acids 128-230), a MR, two C-terminal Aux/IAA dimerization domains, domain III (amino acids 721-756), and domain IV (amino acids 764-807) (Fig. 2-2A). Furthermore, the VvARF7 sequence showed 63 and 61% identity with AtARF7 and SlARF7, respectively, and VvARF8 had 68% identity with AtARF8 and more than 71% identity with SlARF8 (Supplementary Figs. 2-1, 2). Thus, these proteins were renamed VvARF7 and VvARF8 to comply with the nomenclature of the *Arabidopsis* ARFs, based on the similarity between these proteins. Phylogenetic analysis and comparison of VvARF7 and VvARF8 with *Arabidopsis* and tomato ARFs showed that both proteins were clustered with the transcription-activating AtARFs, AtARF5, 6, 7, 8,

A


VvARF7
1
878
DBD
MR
III
IV
VvARF8
1
846
DBD
MR
III
IV

B


AtARF8
VvARF8
SlARF8*
SlARF2
AtARF6
SlARF10
SlARF16
SlARF8
AtARF19
AtARF7
SlARF7*
VvARF7
AtARF5
AtARF3
SlARF3
AtARF4
SlARF14
AtARF17
SlARF13
AtARF10
AtARF16
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AtARF20
AtARF21
AtARF12
AtARF14
AtARF13
AtARF9
SlARF11
SlARF12
AtARF11
AtARF18
AtARF1
SlARF1
AtARF2
SlARF5
SlARF6
0.05

Fig. 2-2. Protein sequence alignment and phylogenetic analysis of VvARF7 and VvARF8. (A) Schematic diagrams of VvARF7 and VvARF8. The length of each protein is denoted. DBD, B3 DNA-binding domain; MR, middle region; III, Aux/IAA dimerization domain III; IV, Aux/IAA dimerization domain IV. (B) Phylogenetic tree of *Vitis* ARF7 and ARF8 with Arabidopsis and tomato ARF families. Asterisks indicate previously reported SlARF7 and SlARF8 proteins, numbered SlARF9 and SlARF4 (Kumar et al., 2012), respectively, based on their locations on the chromosome.

and 19, according to Guilfoyle and Hagen (Guilfoyle and Hagen, 2007) (Fig. 2-2B).

Down-regulation of *VvIAA9* and *VvARF7* with GA application

Using GA applied inflorescence clusters at 14 DBF, which induces parthenocarpy in the seeded diploid 'Tamnara' grapevines, relative transcription levels of *VvIAA9*, *VvARF7*, and *VvARF8* during inflorescence development were analyzed to determine whether GA-induced parthenocarpy in grapevines is associated with auxin-related genes. Without GA application, the expression patterns of *VvIAA9* and *VvARF7* changed similarly until 7 DBF, except for peak expression times of *VvIAA9* and *VvARF7* at 2 DBF and full bloom, respectively, following which the expression levels of both genes rapidly declined (Figs. 2-3A, B). *VvARF8* also showed the highest expression at 2 DBF, with a rapid down-regulation without GA application. With GA application, *VvIAA9* and *VvARF7* expressions at 12 DBF remained at approximately 50 and 14% of the levels observed without GA application, respectively. Furthermore, the expression levels of *VvIAA9* at 2 DBF and *VvARF7* at 5 DBF were significantly lower, dropping to 50 and 37% compared to without GA application, respectively, and *VvARF7* expression further reduced to 15% at full bloom, compared to without GA application (Figs. 2-3A, B). However, *VvARF8* had a fluctuating expression pattern with GA application, showing a more than 2-fold up-regulation from 14 DBF to 5 DAF, and a down-

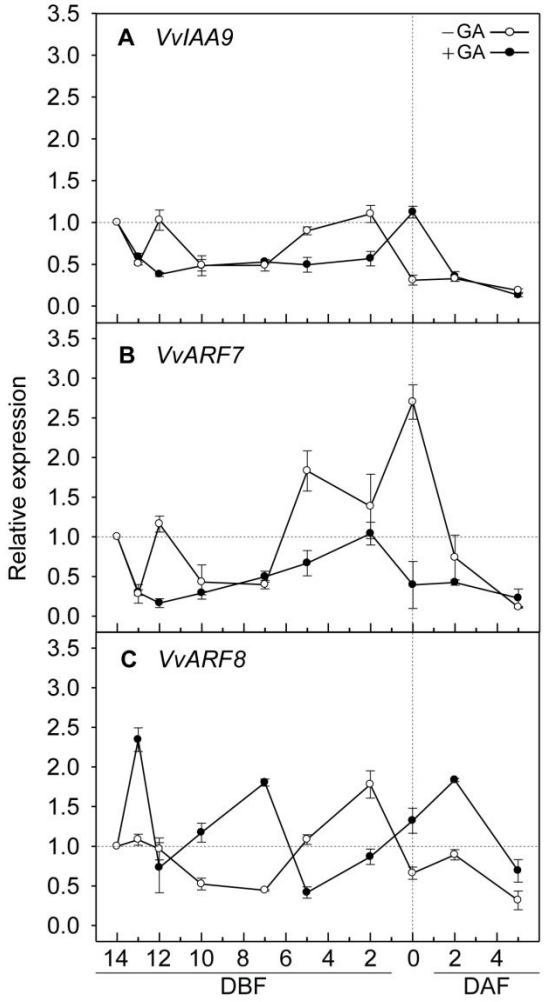

Fig. 2-3. Transcriptional changes in *Vitis* negative regulator genes for fruit set initiation. (A) *VvIAA9*, (B) *VvARF7*, and (C) *VvARF8* in grapevine inflorescences with and without GA application. DAF, days after full bloom; DBF, days before full bloom. qRT-PCR was performed to measure the expression level of each gene using the comparative C_T method. After normalization of each sample to the expression of the internal control *VvActin1*, the expression of each gene relative to the 14 DBF sample was calculated. Bars are standard errors of the means from three independent experiments.

regulation over 20 and 50% at 12 DBF, and during 5 to 2 DBF, respectively, compared to without GA application (Fig. 2-3C). These results indicated that *VvIAA9*, *VvARF7*, and *VvARF8* expressions were maintained at high levels before pollination and rapidly declined after pollination, but GA application at pre-bloom down-regulated *VvIAA9* and *VvARF7* without pollination.

Up-regulation of *VvGH3.2* and *VvGH3.3* with GA application

To determine whether auxin or auxin signaling was affected by GA application in grapevines, the expression patterns of auxin biosynthesis and the auxin-responsive genes were analyzed during inflorescence development. Auxin biosynthesis was verified by analyzing the expression patterns of the *VvASB1-like*, *VvYUC2*, and *VvYUC6* genes, which encode key enzymes in auxin biosynthesis. The expression of *VvASB1-like* was reported by Dauelsberg et al. (2011), and *VvYUC2* and *VvYUC6* were found to be the closest *Vitis* homologue genes of *Arabidopsis YUC2* (*AtYUC2*) and *AtYUC6*, and the tomato *YUC2* homologue, *ToFZY2*, which were abundantly expressed in flowers (Cheng et al., 2006; Expósito-Rodríguez et al., 2011). The VvYUC2 and VvYUC6 proteins shared more than 65% identity and had highly conserved FAD and NADPH binding sites with those of *Arabidopsis* and tomato (Supplementary Figs. 2-3, 4). The levels of *VvASB1-like* transcript fluctuated and peaked at 10 DBF and full bloom, but both *VvYUC2* and *VvYUC6* were expressed at relatively

low levels during inflorescence development without GA application, except for *VvYUC2*, which was up-regulated at 2 DAF (Figs. 2-4A, B, C). With GA application, expression patterns of these auxin biosynthesis genes were not significantly different from those without GA application, except for the up-regulations of *VvASB1-like*, *VvYUC2*, and *VvYUC6* at 7 DBF, full bloom, and 13 DBF, respectively (Figs. 2-4A, B, C). These results indicate that auxin biosynthesis was not affected by GA application. However, transcription levels of the early auxin-responsive genes were significantly changed following GA application. Without GA application, the expression of *VvGH3.2* and *VvGH3.3* declined steadily until 2 DBF and then increased after full bloom, with higher expression of *VvGH3.2* than *VvGH3.3*. With GA application, however, *VvGH3.2* and *VvGH3.3* showed significantly higher levels of transcription at full bloom, with increases of more than 26- and 5-fold, respectively (Figs. 2-4D, E), suggesting that GA application altered auxin signaling.

Up-regulation of *VvDELLA* with GA application

The GA signal was analyzed by monitoring changes in the expression pattern of *VvDELLA* and *VvGAI1*, *Vitis* DELLA family genes, known as key integrators of GA and other hormonal signaling pathways (Boss and Thomas, 2002; Itoh et al., 2002). *VvDELLA* shares 64 and 66% identity with AtGAI and SlDELLA, respectively, and contains highly conserved functional motifs, such as DELLA, TVHYNP, and GRAS domains

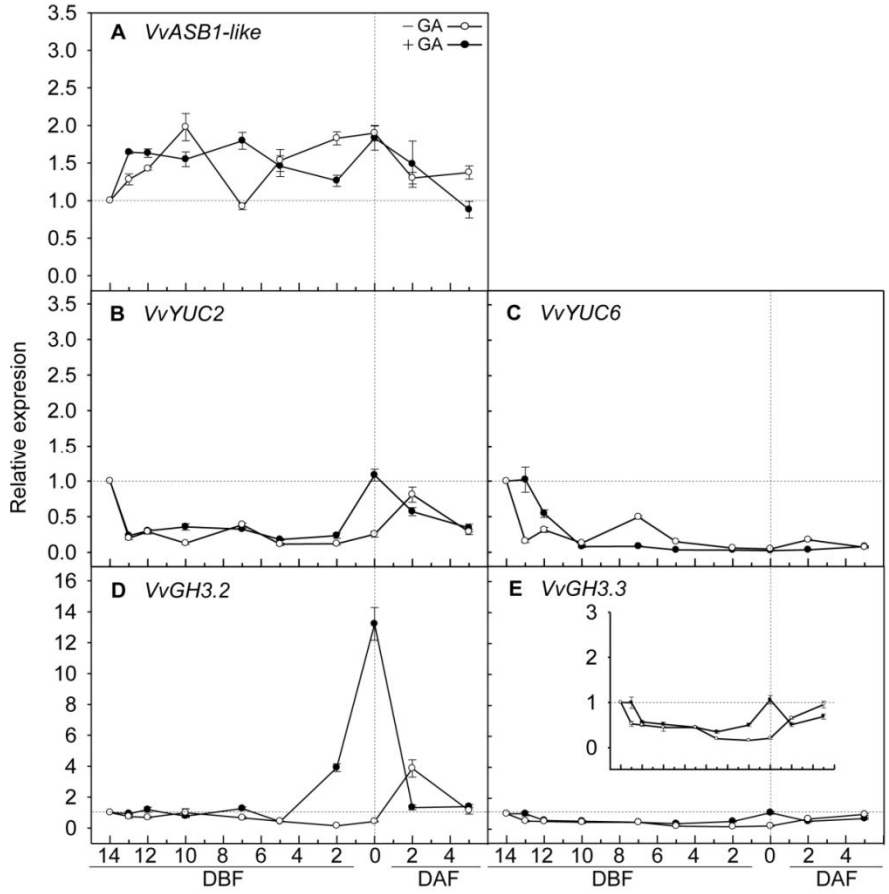

Fig. 2-4. Transcriptional changes in auxin biosynthetic genes (A, B, and C) and early auxin-responsive genes (D and E). (A) *VvASB1-like*, (B) *VvYUC2*, (C) *VvYUC6*, (D) *VvGH3.2*, and (E) *VvGH3.3* in grapevine inflorescences with and without GA application. The expression level of *VvGH3.3* is magnified in the insets. DAF, days after full bloom; DBF, days before full bloom. qRT-PCR was performed to measure the expression level of each gene using the comparative C_T method. After normalization of each sample to the expression of the internal control *VvActin1*, the expression of each gene relative to the 14 DBF sample was calculated. Bars are standard errors of the means from three independent experiments.

(Supplementary Fig. 2-5A, B). Without GA application, transcription levels of *VvDELLA* remained low throughout inflorescence development, and the expression levels of *VvGAI1* were continuously reduced (Fig. 2-5). These expression patterns of *VvGAI1* were not affected by GA application (Fig. 2-5B), but *VvDELLA* showed different expression patterns. One day after GA application, at 13 DBF, the expression level of *VvDELLA* was not significantly different from that observed without GA application. However, expression in *VvDELLA* increased more than 6-fold, from 12 to 7 DBF on GA application, and declined rapidly thereafter (Fig. 2-5A).

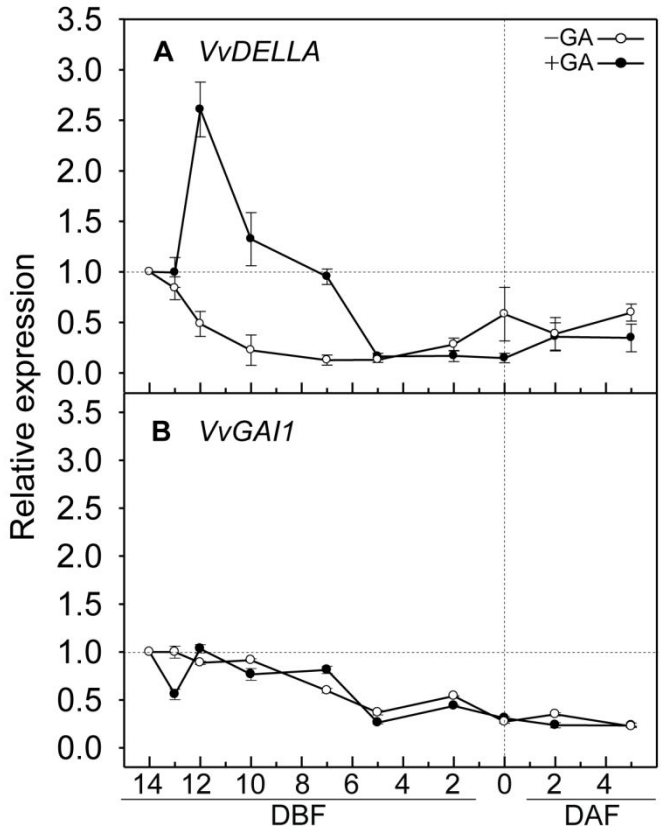

Fig. 2-5. Transcriptional changes in two *DELLA* genes. (A) *VvDELLA* and (B) *VvGAI1* in grapevine inflorescences with and without GA application. DAF, days after full bloom; DBF, days before full bloom. qRT-PCR was performed to measure the expression level of each gene using the comparative C_T method. After normalization of each sample to the expression of the internal control *VvActin1*, the expression of each gene relative to the 14 DBF sample was calculated. Bars are standard errors of the means from three independent experiments.

DISCUSSION

GA application induces parthenocarpy by early down-regulation of negative regulators of fruit set initiation

Parthenocarpic fruit development in mutants of the *IAA9*, *ARF7*, and *ARF8* genes was the result of reduced expression of these genes before pollination (de Jong et al., 2009b; Goetz et al., 2006; 2007; Wang et al., 1993). Without GA application, *VvIAA9*, *VvARF7*, and *VvARF8* were highly expressed until near full bloom, after which their expressions declined after 2 DBF or full bloom (Fig. 2-3). These results imply that *VvIAA9*, *VvARF7*, and *VvARF8* function as negative regulators of fruit set initiation, whose down-regulation leads to fruit set initiation. With GA application, however, *VvIAA9* and *VvARF7* were down-regulated 2 days after the application, and their transcription levels remained low until full bloom or 2 DBF, respectively (Figs. 2-3A, B), in which expression levels of GA biosynthesis genes were down-regulated by GA application at 14 DBF (Jung et al., 2014). These results indicate that effects of GA application at pre-bloom were not limited on transcriptional changes of these genes at 1 or 2 days after the application. An enlarged ovary and inhibition of pollen tube growth were observed, both in the pistil of grapevines with GA applied at 14 DBF (Jung et al., 2014; Okamoto and Miura, 2005), and in *ARF7*-silencing lines (de Jong et al., 2009b), substantiating the down-regulatory effects of GA application on *VvARF7* expression at full bloom.

These results also suggest that GA can substitute for the effects of auxin in fruit set initiation in the absence of pollination. Although *ARF8* has been reported as a negative regulator of fruit set initiation in *Arabidopsis* and tomato (Goetz et al., 2006; 2007), only truncated, and not null mutants of *ARF8* showed parthenocarpic fruit initiation (Goetz et al., 2006), and direct involvement of *ARF8* in parthenocarpic fruit development was not observed in the present study.

To verify whether reduced transcription of *VvIAA9* and *VvARF7* with GA application was due to an increase in auxin levels, transcriptional changes were monitored in the auxin biosynthesis genes, *VvASB1-like*, *VvYUC2*, and *VvYUC6*, and two early auxin-responsive gene, *VvGH3.2* and *VvGH3.3*. *VvASB1-like* encodes the first step enzyme of auxin biosynthesis (Stepanova et al., 2005), and two *Vitis YUC* genes; *VvYUC2* and *VvYUC6* genes, encode the final step enzymes (Mashiguchi et al., 2011). An accumulation of auxin, resulting from the up-regulation of these genes, has been reported in *Arabidopsis* (Kim et al., 2011; Mano and Nemoto, 2012; Mashiguchi et al., 2011; Stepanova et al., 2005). The up-regulation of *VvASB1-like* upon pollination has also been reported in grapevines (Dauelsberg et al., 2011). Furthermore, auxin or auxin signaling mediated induction of the *GH3* gene family, which encode the early auxin-responsive IAA-amino synthetases, have been reported in *Arabidopsis* (Nakazawa et al., 2001; Staswick et al., 2005) and tomato (de Jong et al., 2009b; 2011). In grapevines, six *Vitis GH3* family genes have

been identified, and both *VvGH3.2*, the most abundantly expressed *Vitis GH3* gene in flower and *VvGH3.3*, the closest homolog of *AtGH3.6* in *Vitis* (Böttcher et al., 2011), showed auxin-inducible expression patterns. With GA application, there was only a slight fluctuation of the expression patterns of *VvASB1-like*, *VvYUC2*, and *VvYUC6* (Figs. 2-4A, B, C), however *VvGH3.2* and *VvGH3.3* were up-regulated more than 3-fold between 2 DBF and full bloom (Figs. 2-4D, E), when *VvIAA9* and *VvARF7* were down-regulated. With GA application, *VvGH3.2* was expressed 4-fold higher at full bloom, compared to without GA application at 2 DAF (Fig. 2-4D), indicating that the up-regulation of *VvGH3.2* at full bloom was due to the GA application rather than to ovary development. These results are in agreement with the observed up-regulation of the *SlGH3-like* gene expression in a silencing line of *SlARF7* (de Jong et al., 2009b), and also support the idea that GA application down-regulates *VvARF7*, with partial activation of auxin signaling during parthenocarpic fruit development. However, the possibility of GA-mediated accumulation of auxin could not be excluded, since the peak expression time of *VvYUC2* correlated with the highest expression of *VvGH3.2* at full bloom with GA application (Figs. 2-4B, D).

Integration of GA and auxin signalings during parthenocarpic fruit development via *VvDELLA*

The crosstalk between GA and auxin in fruit set initiation has been

demonstrated by the effects of auxin on GA biosynthesis in *Arabidopsis* and tomato (Dorcey et al., 2009; Serrani et al., 2008), and the partial involvement of GA signaling in silencing lines of *ARF7* in tomato (de Jong et al., 2009b; 2011). Using the *pro* tomato mutant, a loss-of-function mutant of *SlDELLA*, Carrera et al. (2012) demonstrated that GA affected a component of auxin signaling by down-regulating the negative regulators of fruit set initiation; *SlIAA9* and *SlARF7*. Thus, GA and auxin may activate each other in the signaling pathway to a certain extent, and this integration appears to be associated with parthenocarpy induction. The activation of auxin signaling by GA application observed in this study was consistent with the partial auxin signaling activation in silencing lines of *SlARF7* (de Jong et al., 2009b). Furthermore, with GA application, the prior up-regulation of *VvDELLA* decreased of expression levels of *VvIAA9* and *VvARF7*, suggesting that GA application induced parthenocarpic fruit development by reducing *VvIAA9* and *VvARF7* via *VvDELLA* (Figs. 2-3A, B; 2-5A). The up-regulation of *VvDELLA* indicated that its transcription was under a GA-mediated feedback regulation, as observed in *SlDELLA* (Carrera et al., 2012). However, *VvGAI1*, another *Vitis* DELLA family gene, identified previously as a floral induction related *Vitis* DELLA family gene (Boss and Thomas, 2002), did not show transcriptional changes, regardless of GA application, as did the *Arabidopsis* DELLA family genes. These differential transcriptional regulations by GA application between *VvDELLA* and *VvGAI1* may have originated from the differences in the

amino acid sequences of the Poly S/T/N motif, the transcriptional regulatory region of DELLA proteins (Itoh et al., 2002; Sheerin et al., 2011; Sun, 2008). A comparison of the amino acid sequences of the Poly S/T/N motif showed that VvDELLA and SlDELLA shared more conserved amino acids than VvGAI1 and the amino acid sequences of VvGAI1 in this motif were similar to those of AtGAI and AtRGA (Supplementary Fig. 2-5C, D).

Based on the results presented in this study and previous data from de Jong et al. (2011) and Carrera et al. (2012), a model for fruit set initiation is proposed to be mediated by *VvARF7*, *VvIAA9*, and *VvDELLA* in grapevines with or without GA application. Elevated GA and auxin upon pollination initiate fruit set by down-regulating *VvIAA9* and *VvARF7*, negative regulators of fruit set initiation (Fig. 2-6A). With GA application, however, the consecutive transcriptional changes in *VvDELLA*, *VvIAA9*, and *VvARF7*; i.e., the up-regulation of *VvDELLA* followed by the down-regulation of *VvIAA9* and *VvARF7* during inflorescence development (Figs. 2-3A, B, 2-5D), and the early reduction of *VvIAA9* and *VvARF7* may replace the effects of auxin and initiate parthenocarpic fruit development (Fig. 2-6B).

In this study, the complex expression patterns of fruit set initiation related genes were reviewed in grapevines and GA application at pre-bloom was shown to down-regulate *VvIAA9* and *VvARF7* before pollination and to activate some auxin signaling via *VvDELLA* during parthenocarpic fruit development. This is the first report to detail the

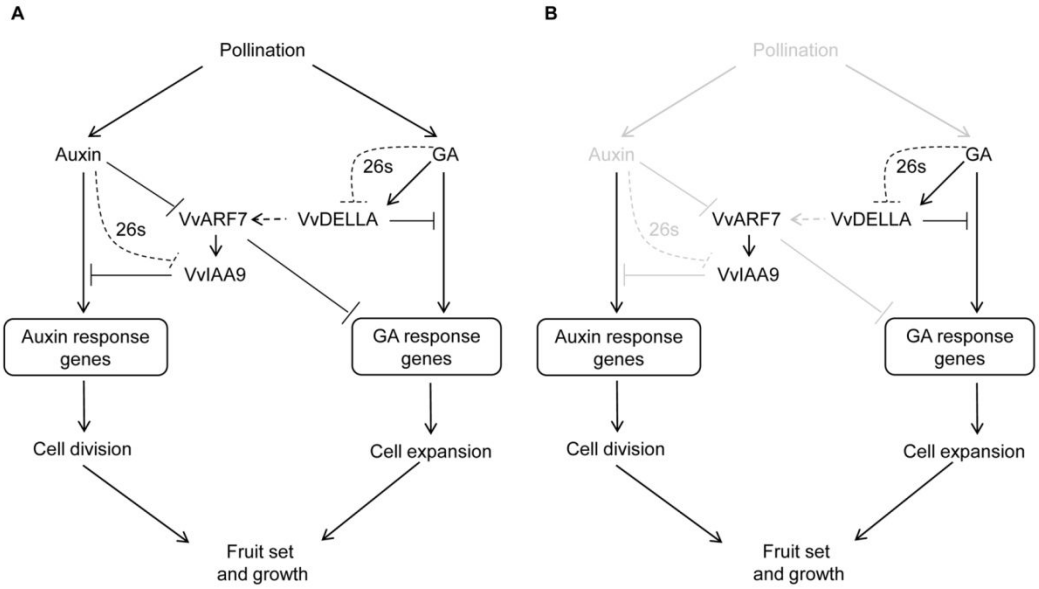

Fig. 2-6. Proposed model for GA and auxin crosstalk in grapevines during fruit set initiation. The possible functions of *VvARF7*, *VvIAA9*, and *VvDELLA* are also included, based on de Jong et al. (2011) and Carrera et al. (2012). (A) Pollination-mediated fruit set initiation. The dashed line with 26S indicates the regulation of protein levels by 26S proteasome-mediated degradation, and the arrow indicates activation at the protein level. (B) Putative hormone signaling in fruit set initiation by GA application on grapevine inflorescences. Pathways inactivated by GA application are indicated in gray.

molecular mechanism of fruit set initiation in grapevines, and contributes to improving fruit productivity by providing information to increase fruit set initiation of other important crops. The integration of GA and auxin signaling, including the roles of *VvIAA9*, *VvARF7*, and *VvDELLA* should further be investigated to broaden our understanding of the molecular mechanisms underlying GA-mediated induction of parthenocarpic fruit development in viticulture.

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CONCLUSIONS

In viticulture, gibberellins (Gas) have commonly been employed to induce parthenocarpy. Although GA-mediated parthenocarpy is a highly desirable trait for table grapes, the molecular mechanism underlying this induction is poorly understood. GA was applied to inflorescence clusters of seeded diploid grapevine cultivar 'Tamnara' (*Vitis* spp.) at 14 days before full bloom (DBF). The morphological and molecular effects on the induction of parthenocarpic fruit development were examined following GA application.

GA application to grapevine inflorescence at pre-bloom increased ovary and ovule size, arrested pollen tube growth, and induced parthenocarpic fruit development. *Vitis* GA oxidase enzymes, key determinants for GA level, were characterized through phylogenetic analysis with *Arabidopsis* GA oxidase enzymes. Five VvGA 20-oxidase (VvGA20ox), three VvGA 3-oxidase (VvGA3ox), and nine VvGA 2-oxidase (VvGA2ox) family proteins, and one VvGA methyltransferase (VvGAMT) and one *Vitis* cytochrome P450 (VvCYP) 714A protein were identified, and their expression patterns were analyzed during inflorescence development from 14 DBF to 5 days after full bloom (DAF). *VvGA2ox1*, *VvGA20ox3*, and *VvGA3ox2* were the most abundantly expressed genes in each gene family at 7, 5, and 2 DBF, respectively. Following GA application at 14 DBF inducing seedlessness, GA catabolic genes such as *VvGAMT2,*

VvGA2ox3, and *VvGA2ox4* were up-regulated at 12 DBF, full bloom, and 5 DAF, respectively. Conversely, most GA biosynthetic genes, *VvGA20oxs* and *VvGA3oxs*, were down-regulated at near full bloom, and the timing of their peak expression was changed. These results suggest that GA application at pre-bloom changes the GA biosynthesis into GA catabolic pathway at near full bloom by altering the transcription level and timing of GA oxidase genes during grapevine inflorescence development.

The transcriptional levels of the putative negative regulators of fruit set initiation, including *Vitis auxin/indole-3-acetic acid transcription factor 9* (*VvIAA9*), *Vitis auxin response factor 7* (*VvARF7*), and *VvARF8* were also monitored during inflorescence development. Without GA application, *VvIAA9, VvARF7*, and *VvARF8* were expressed at a relatively high level before full bloom, but decreased thereafter following pollination. After GA application at 14 DBF, however, the expression levels of *VvIAA9* and *VvARF7* declined at 5 DBF prior to pollination. The effects of GA application on auxin levels or auxin signaling were also analyzed by monitoring the expression patterns of auxin biosynthesis genes and auxin-responsive genes with or without GA application. Transcription levels of the auxin biosynthesis genes *Vitis anthranilate synthase β subunit* (*VvASB1-like*), *Vitis YUCCA2* (*VvYUC2*), and *VvYUC6* were not significantly changed by GA application. However, the expressions of *Vitis Gretchen Hagen3.2* (*VvGH3.2*) and *VvGH3.3*, auxin-responsive genes, were up-regulated from 2 DBF to full bloom with GA application.

Furthermore, the *Vitis* GA signaling gene, *VvDELLA* was up-regulated by GA application during 12 DBF to 7 DBF, prior to down-regulation of *VvIAA9* and *VvARF7*. These results suggest that *VvIAA9* and *VvARF7* are negative regulators of fruit set initiation in grapevines, and GA signaling is integrated with auxin signaling via *VvDELLA* during parthenocarpic fruit development in grapevines.

With GA application, therefore, fertilization could not occur due to the inhibited pollen tubes, but ovaries enlarged and developed into parthenocarpic fruit due to the down-regulation of *VvIAA9* and *VvARF7* before pollination and activated a part of auxin signaling via *VvDELLA* during parthenocarpic fruit development. This report is the first to detail the molecular mechanism of fruit set initiation in grapevine and should contribute to improving fruit productivity by providing the clue to increase fruit set initiation of other important crops. The integration of GA and auxin signaling, including the roles of *VvIAA9*, *VvARF7*, and *VvDELLA* should further be investigated to broaden our understanding of the molecular mechanisms underlying GA-mediated induction of parthenocarpic fruit development in viticulture.

ABSTRACT IN KOREAN

유핵 포도 품종에 지베렐린을 처리하여 단위결과성의 무핵 포도를 생산하는 기술은 1960년대부터 개발되어 실용화된 기술이지만, 이에 대한 분자생물학적 작용 기작 연구는 거의 이루어지지 않았다. 지베렐린 처리로 유도되는 포도 단위결과의 분자생물학적 작용 기작을 구명하기 위해 지베렐린 대사 및 옥신 신호 전달과 관련된 유전자들을 동정하고 과실 발달 과정의 시기별 발현량을 조사하였다. 2배체, 유핵 품종인 '탐나라(*Vitis* spp.)' 화뢰에 만개 14일 전에 지베렐린을 처리하여 단위결과를 유도하였다.

개화 전에 지베렐린을 처리하면 씨방은 비대하나 꽃가루관 신장이 저해되어 수정이 일어나지 않았다. 세포 내 지베렐린의 농도를 결정하는 것으로 알려진 *Vitis* GA oxidases는 *Arabidopsis* GA oxidases를 이용한 계통 분석(phylogenetic analysis)을 통해, 5개의 VvGA 20-oxidase(VvGA20ox), 3개의 VvGA 3-oxidase (VvGA3ox), 그리고 9개의 VvGA 2-oxidase(VvGA2ox) family proteins과 1개의 VvGA methyltransferase(VvGAMT), 1개의 *Vitis* cytochrome P450(VvCYP) 714A 단백질을 동정하였으며, 만개 14일 전부터 만개 5일 후까지 화기 발달 과정 동안 각 유전자들의 발현 양상을 분석하였다. *VvGA2ox1*, *VvGA20ox3*, *VvGA3ox2*는 만개 7일, 5일, 2일 전에 각각의 유전자군에서 가장 높게 발현하였다. 만개 14일 전에 지베렐린을 처리하면 무핵과가 생산되는데, *VvGAMT2, VvGA2ox3*, *VvGA2ox4*과 같은 GA 생분해 유전자들의 발현이 각각 만개 12일 전, 만개, 만개

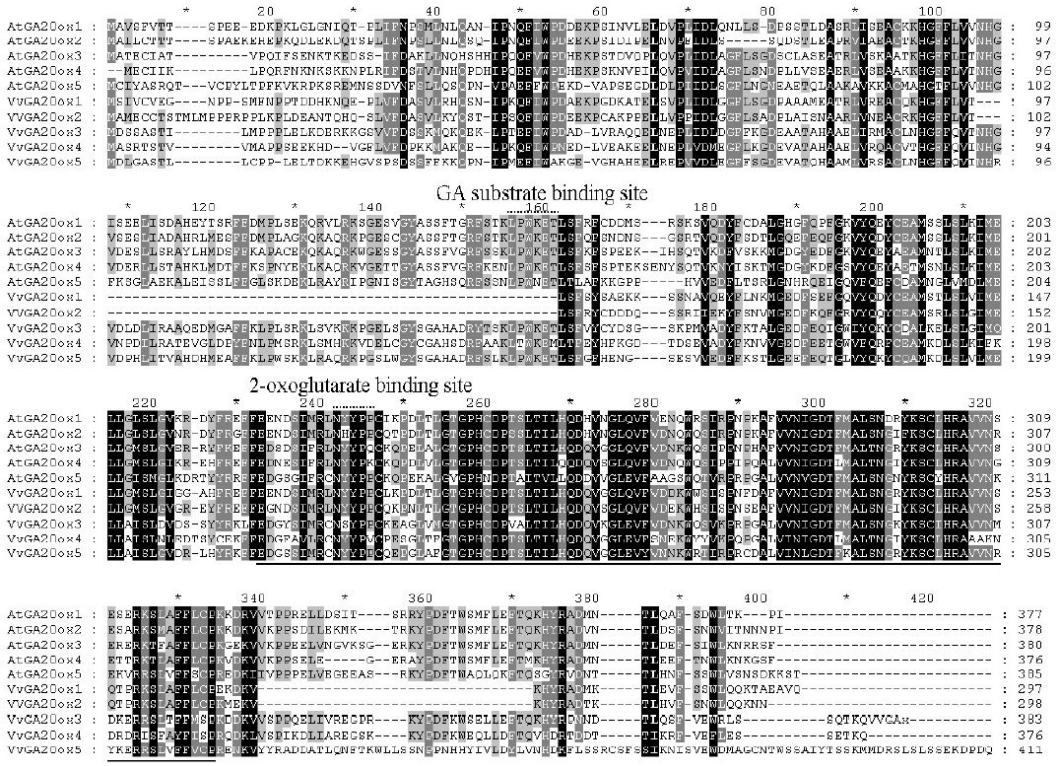
5일 후에 증가하였다. 반면, 지베렐린 생합성 유전자인 *VvGA20oxs*와 *VvGA3oxs*의 발현은 만개기 근처에서 감소하였고, 발현이 가장 높은 시기가 변하였다. 이러한 결과는 개화 전에 지베렐린을 처리하면, 화기 발달 과정 중의 지베렐린 대사와 관련된 유전자들의 발현량과 발현 시기에 영향을 끼쳐 개화기를 전후하여 지베렐린 생합성 과정에서 지베렐린 생분해 과정으로의 변화가 일어난다는 것을 관찰하였다.

착립과 과실 발달의 역조절인자(negative regulator)로 알려진 *Vitis auxin/indole-3-acetic acid transcription factor 9*(*VvIAA9*), *Vitis auxin response factor 7*(*VvARF7*), *VvARF8*의 발현을 화기 발달 과정 동안 분석하였다. 지베렐린을 처리하지 않았을 때, *VvIAA9, VvARF7*, *VvARF8*는 만개 전에 비교적 높게 발현하다가 수분이 이루어진 이후에 감소하였다. 그러나 만개 14일 전에 지베렐린을 처리하면 *VvIAA9*와 *VvARF7*의 발현은 수분이 이루어지기 전인 만개 5일 전부터 감소하고, 이로 인해 수정이 되지 않은 과실도 탈립되지 않고 비대하여 단위결과성 과실로 발달한다는 사실을 관찰하였다. 지베렐린 처리가 옥신의 생합성에 영향을 끼치는지 아니면 옥신 신호 전달에만 영향을 끼치는지 알아보기 위해, 지베렐린 처리구와 대조구에서 시기별로 옥신 생합성 유전자와 옥신 반응(auxin-responsive) 유전자의 발현을 분석하였다. 옥신 생합성 유전자인 *Vitis anthranilate synthase β subunit*(*VvASB1-like*), *Vitis YUCCA2*(*VvYUC2*), *VvYUC6*의 발현은 지베렐린 처리에 의한 유의한 변화가 일어나지 않았으나 옥신 반응(auxin-responsive) 유전자인 *Vitis Gretchen Hagen3.2*(*VvGH3.2*)와 *VvGH3.3*의 발현은 만개 2일 전부터 만개기까지 증가하는 경향이었다.

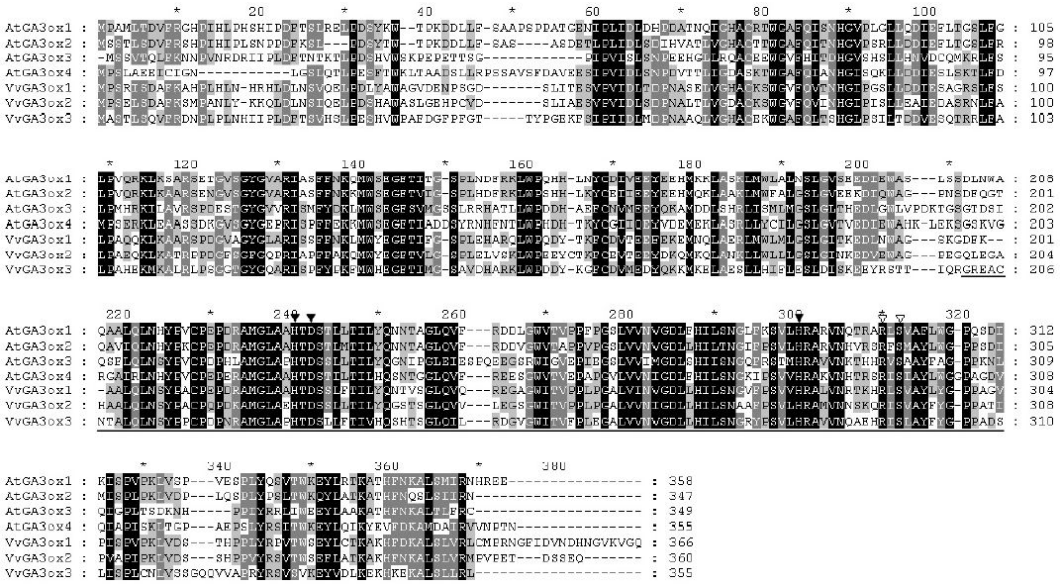
또한, *Vitis* GA 신호 유전자인 *VvDELLA*의 발현은 *VvIAA9*와 *VvARF7*의 발현이 감소하기 전인 만개 12일 전부터 7일 전까지 급격히 증가하였다. 따라서 *VvIAA9*와 *VvARF7*는 포도에서 착립 개시와 과실 발달에 관여하는 역조절인자로 작용하며, 지베렐린 처리에 의해 단위결과성 과실이 발달하는 과정에 지베렐린과 옥신의 신호 전달에 VvDELLA가 관여하고 있음을 알 수 있었다.

Supplementary Table 1-1. Primers used for qRT-PCR.

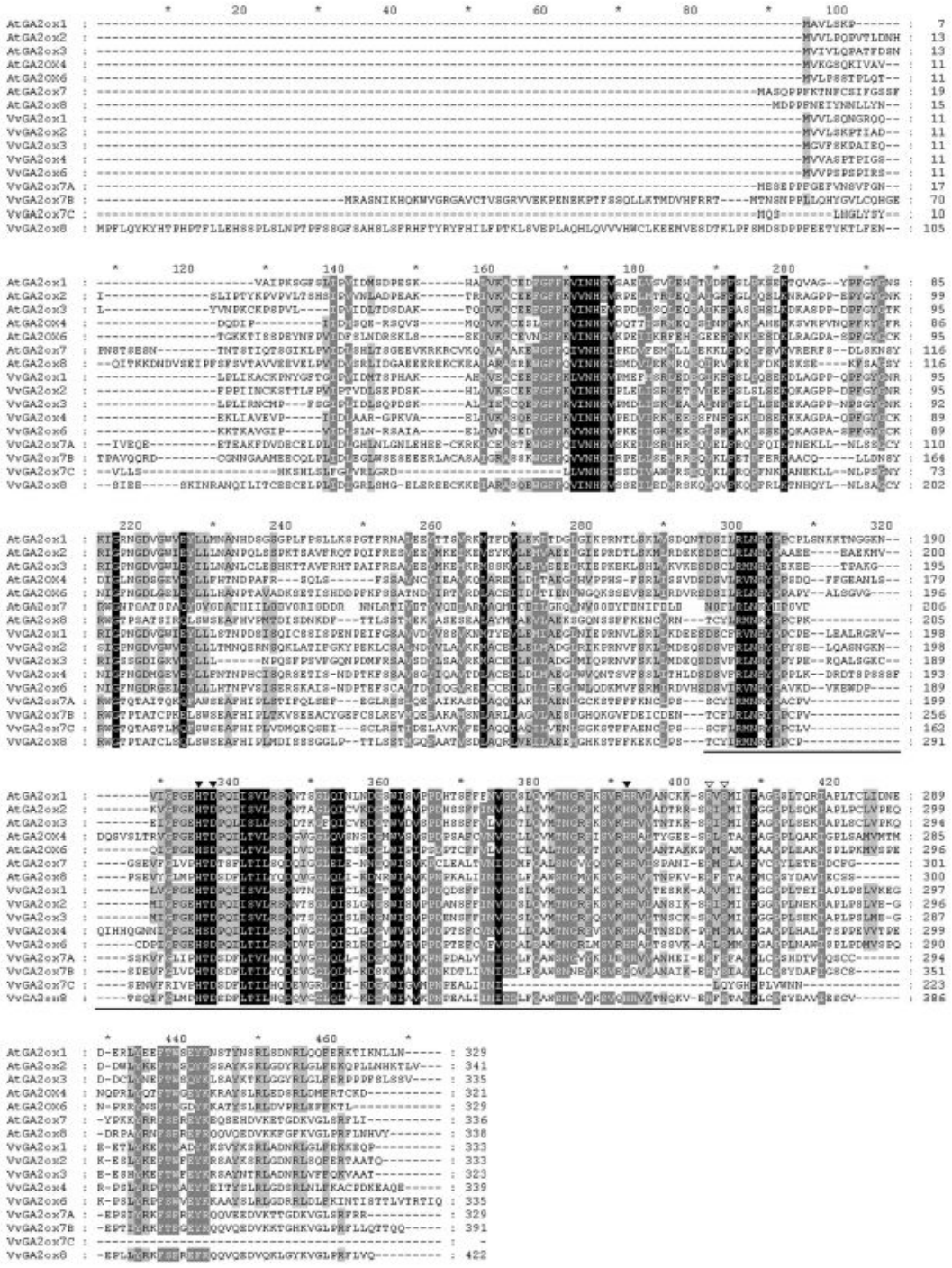
Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
VvGA20ox1	GGATAGGAATGACGCAACAGTGAATTTC	GGAAGTCGGTGAACAAAAATTGGAATC
VvGA20ox2	CCCCAATGAACGGCCTTGG	GGAGAGGAAGAAGGGAGTGTGAAATAAG
VvGA20ox3	GGTCAGAATTGTTGGAGTTCACTCAGAAG	CCACAAAGCTCTGGAGAGTATCATTATTGG
VvGA20ox4	CGTTGGATTTTTAGTTTTTGACCCAAAG	GTTTGGGAAGCTCTTGTTTTGCCAT
VvGA20ox5	ATTTCAGTTGAGTGGGATATGGCGG	GAGCTTGTGTATATAGCACTTGACCAAGTG
VvGA3ox1	CCTATGTTTTCTCCATAACACAAGTACTACCAC	GGGAGAAGTGGGGGGTGTTGG
VvGA3ox2	GTTTGGCCATGTCGTGTAGGACTCTC	CCATTTCTTCATCACTCTCTTATTACCACCA
VvGA3ox3	GTAGAGGAAGAAGAAGAAGAAGATGATGATGAT	TCTCCACCTCCACCCCCTCC
VvGA2ox1	GGGCTCTTTGAGAAAAAAGAGCAGC	CCATAGGTTTTCCTAAAATGGGGAAATT
VvGA2ox2	CTTAAGCATTTCCTCGTACGACCTCTG	GGTTGCTATGAAGAAATAAATGGAACGAT
VvGA2ox3	GCCTCCGCTCAAACAATACCAACTC	GGGTATTGGGTGTTGATGATATTAAGAAATG
VvGA2ox4	GCCAAATTTTGCTTATAAGTTTCCGCTA	CCATTTCTTCTGTATACACACACTCATCCAG
VvGA2ox6	TTTCAATCTTTCTCTCTTCTAATCACACAATTATG	GGCAGACCTATTGAGAGAAAGGTCTATTACTG
VvGA2ox7A	GCCCCTTATTCCACCCTAAACCAC	AGTTGCAATAGATGAGTGTATACAATAGATGCG
VvGA2ox7B	GAGTGGTTGAGAAACCAGAAAATGAAAAG	TGGACATCCATTGTTTTCAGGAGTTG
VvGA2ox7C	GAAACCCTAAAGATAGTTATAGGGGCCTTATC	CATACCATGTGAAAGAGTATTTCATTCAGAGG
VvGA2ox8	CCATTCCCACAAAATCTCAGTACCACTG	CTATACCCTTAGTTGCTTTGGCATTCAAA
VvGAMT2	GGACTCATCTAGTTCAATGGCCATATCC	CGGCTGGCACTCCCTTTTCATG
VvCYP714A1	GCAGTAAAAGAGCGAGAACAAGAATGCT	TCATTGATGGCACTCTCCAGTAGCAG
Vvactin1	GCTGGATTCTGGTGATGGTG	CCAATGAGAGATGGCTGGAA



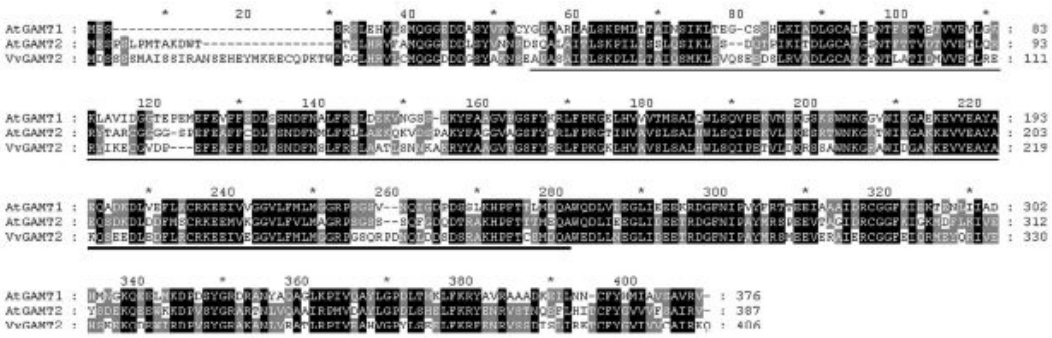
Supplementary Fig. 1-1. Sequence analysis of VvGA20ox family proteins. Comparison of VvGA20oxs and AtGA20oxs. Both consensus sequences LPWKET for GA substrates binding and NYYPXCXXP for the common co-substrate, 2-oxoglutarate, binding of *Vitis* GA20oxs are denoted with dotted lines. The $F2^{2+}$ 2-ODD domain is underlined with a solid line.



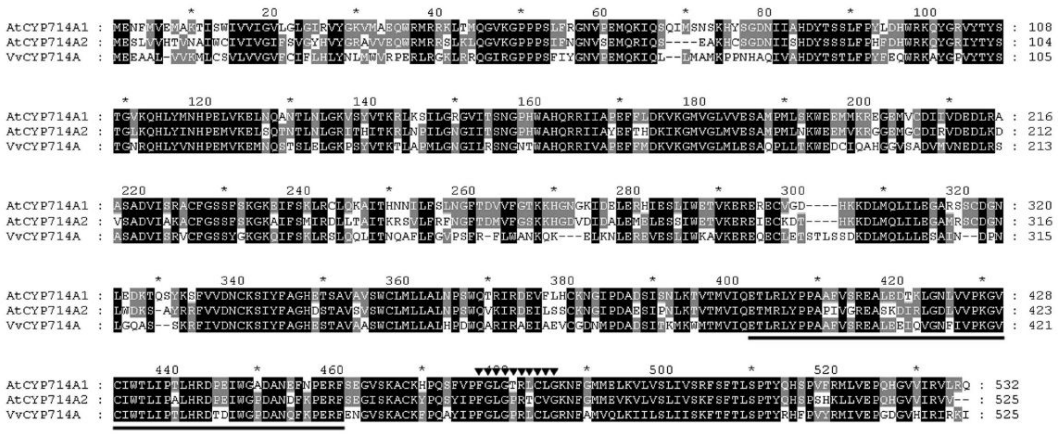
Supplementary Fig. 1-2. Sequence analysis of VvGA3ox family proteins. Comparison of VvGA3oxs and AtGA3oxs. ▼, Fe^{2+} binding site; ▽, 2-oxoglutarate binding site. The Fe^{2+} 2-ODD domain is underlined with a solid line.


AtGA2ox1
AtGA2ox2
AtGA2ox3
AtGA2ox4
AtGA2ox6
AtGA2ox7
AtGA2ox8
VvGA2ox1
VvGA2ox2
VvGA2ox3
VvGA2ox4
VvGA2ox6
VvGA2ox7A
VvGA2ox7B
VvGA2ox7C
VvGA2ox8

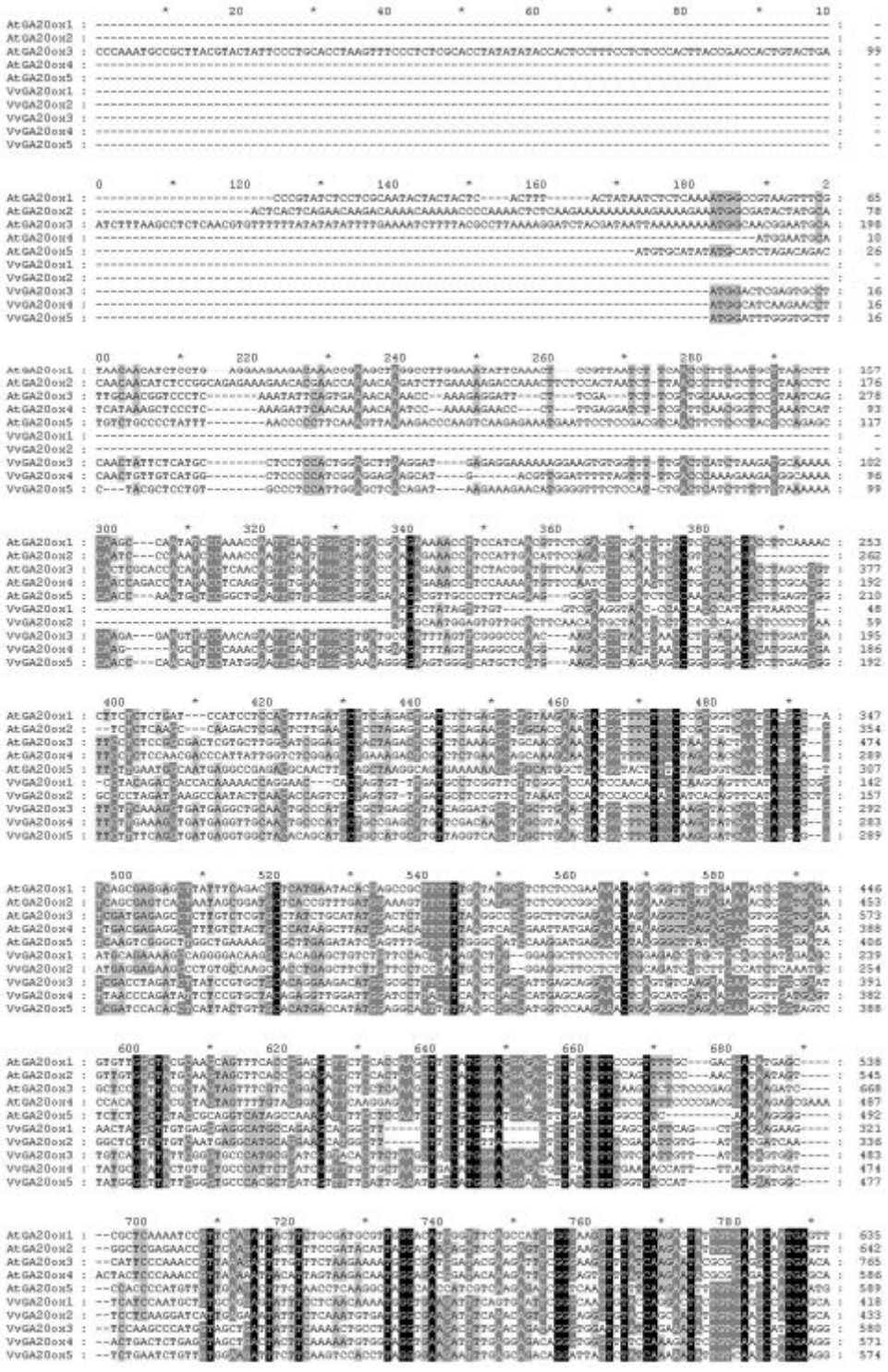
Supplementary Fig. 1-3. Sequence analysis of VvGA2ox family proteins. Comparison of VvGA2oxs and AtGA2oxs. ▼, Fe^{2+} binding site; ▽, 2-oxoglutarate binding site. The Fe^{2+} 2-ODD domain is underlined with a solid line. ▼, heme binding site. The conserved domain is underlined. Highly conserved amino acids are in black and similar amino acids are gray colored.

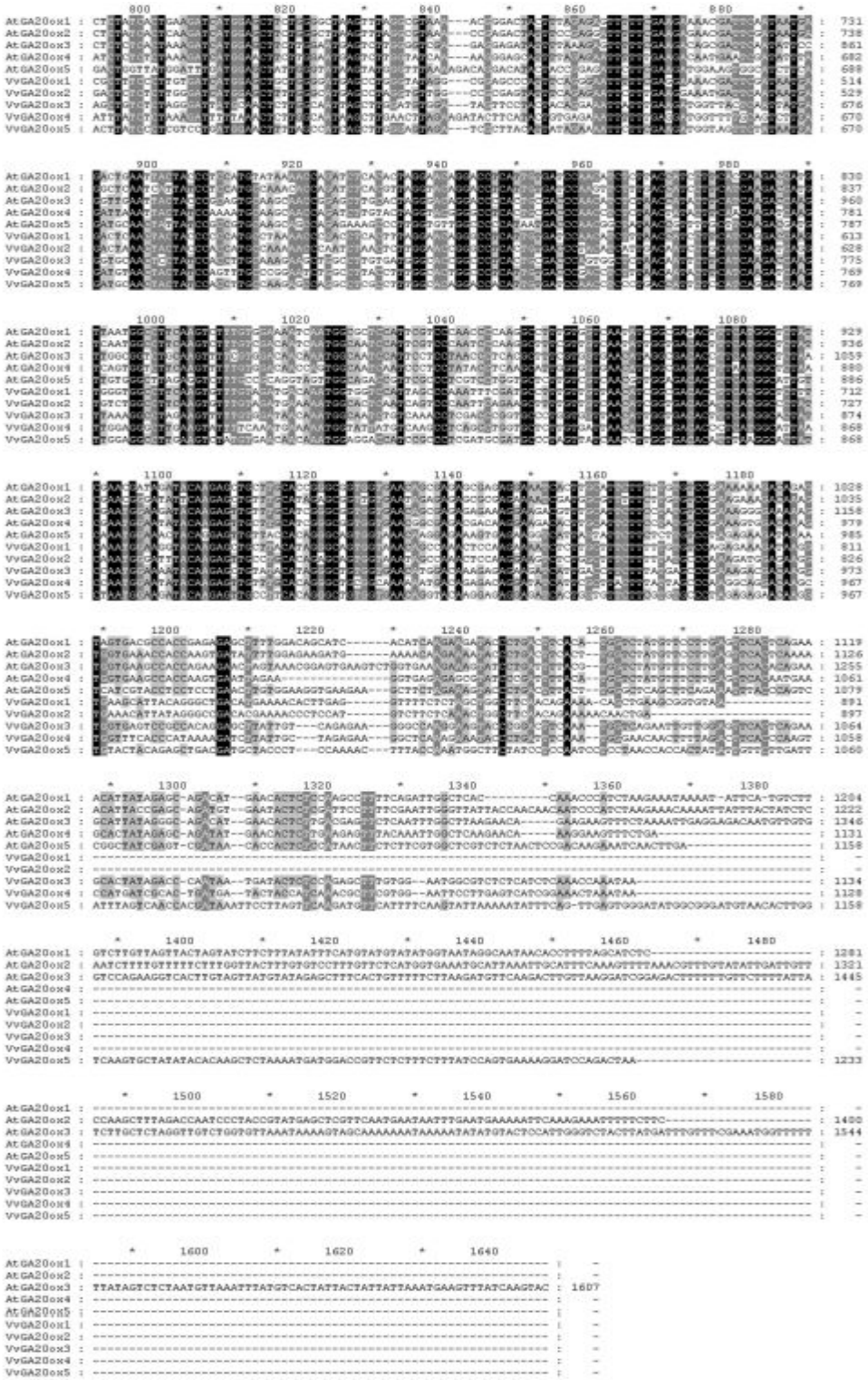


Supplementary Fig. 1-4. Sequence analysis of VvGAMT2 protein. Comparison of VvGAMT2 with AtGAMT1 and AtGAMT2. The S-adenosyl-L-methionine-dependent methyltransferase domain is underlined. The conserved domain is underlined. Highly conserved amino acids are in black and similar amino acids are gray colored.

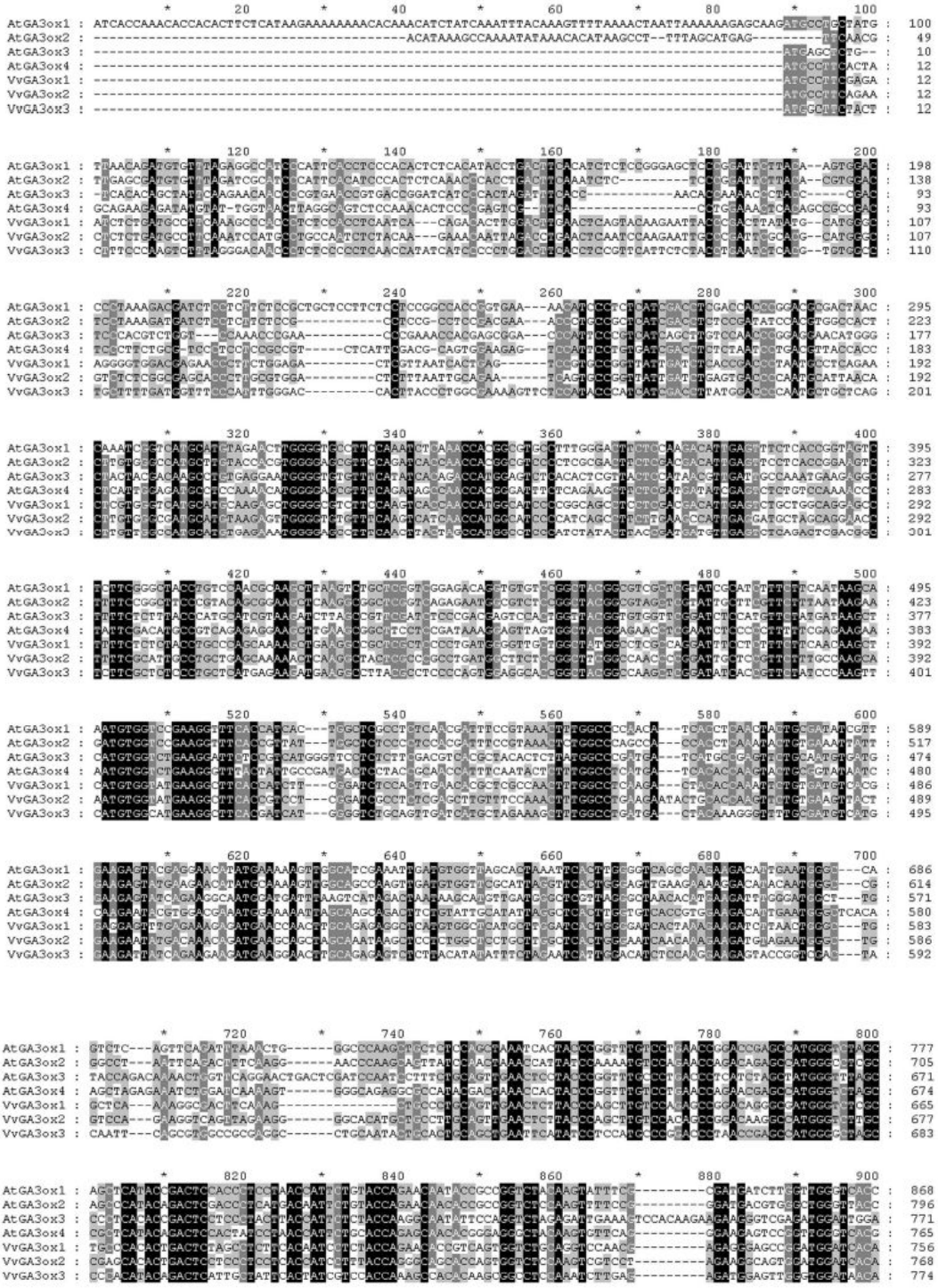


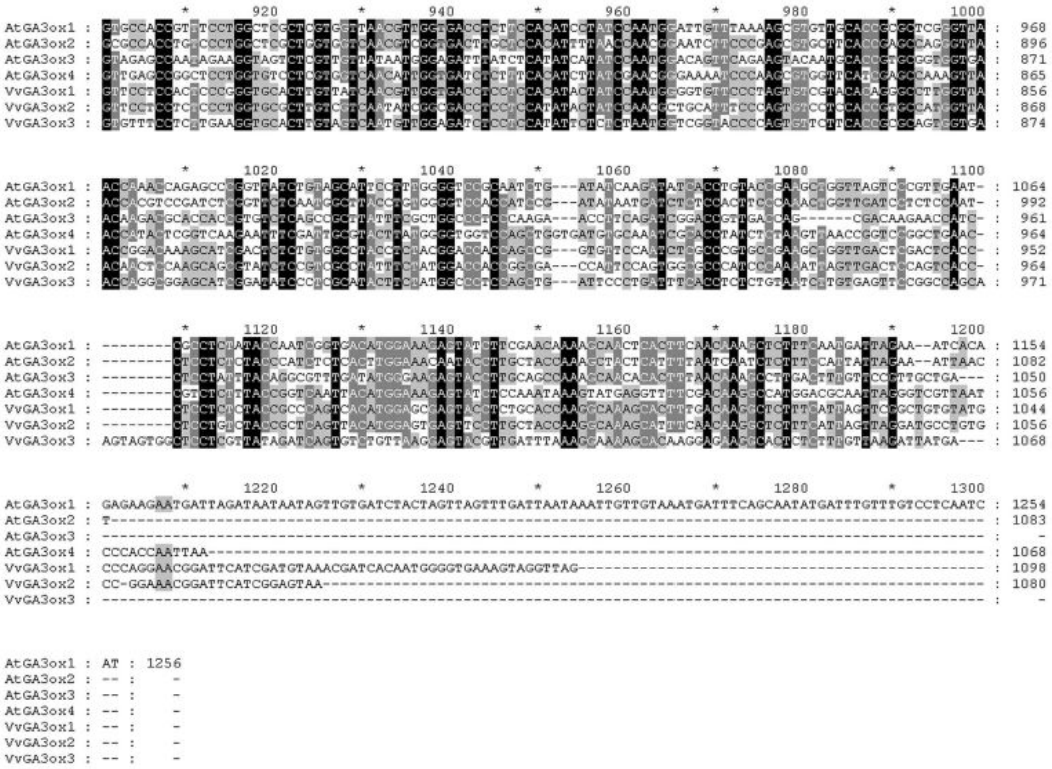
Supplementary Fig. 1-5. Sequence analysis of VvCYP714A protein. Comparison of VvCYP714A, AtCYP714A1, and AtCYP714A2. ▼, heme binding site. The conserved domain is underlined. Highly conserved amino acids are in black and similar amino acids are gray colored.



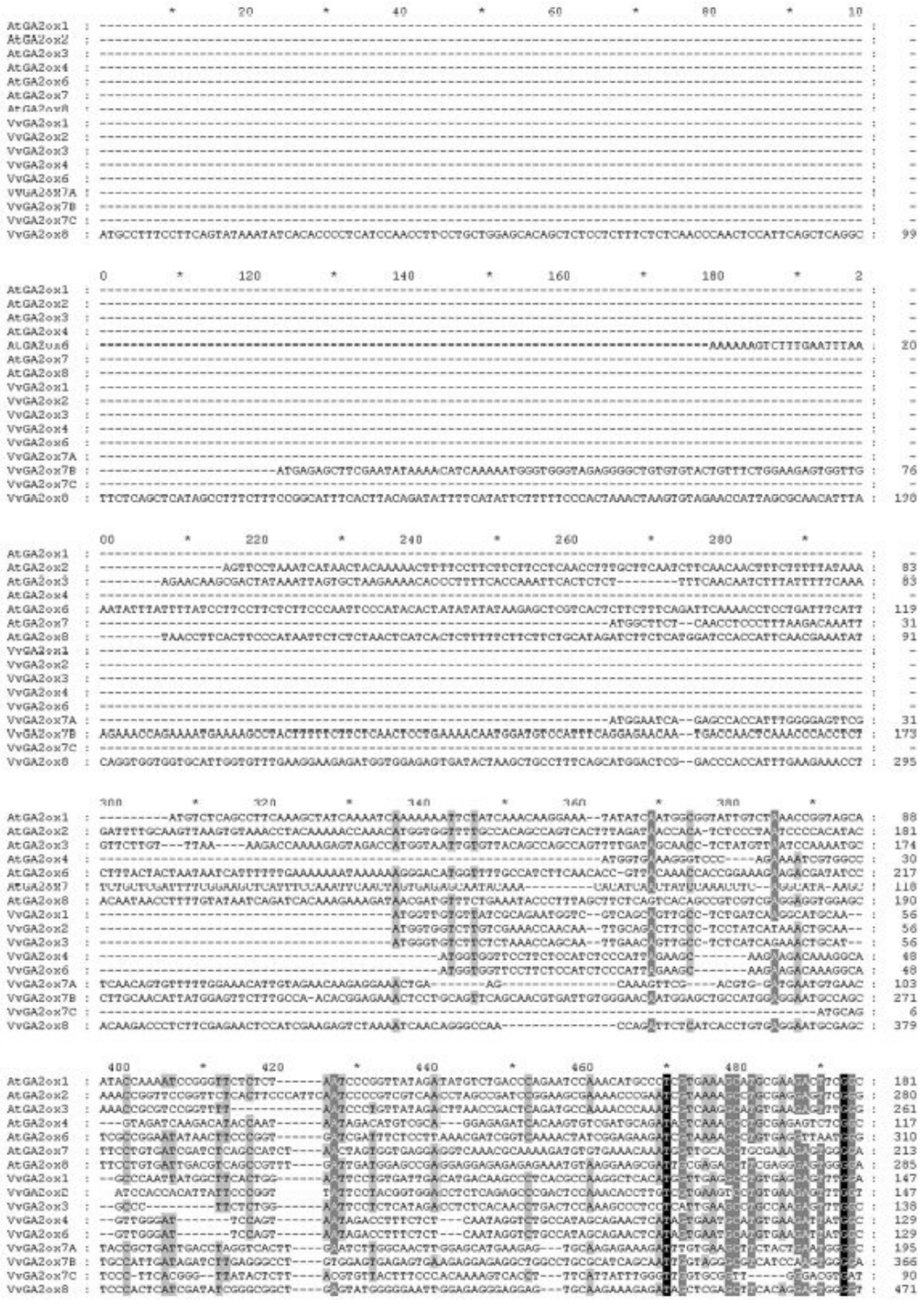


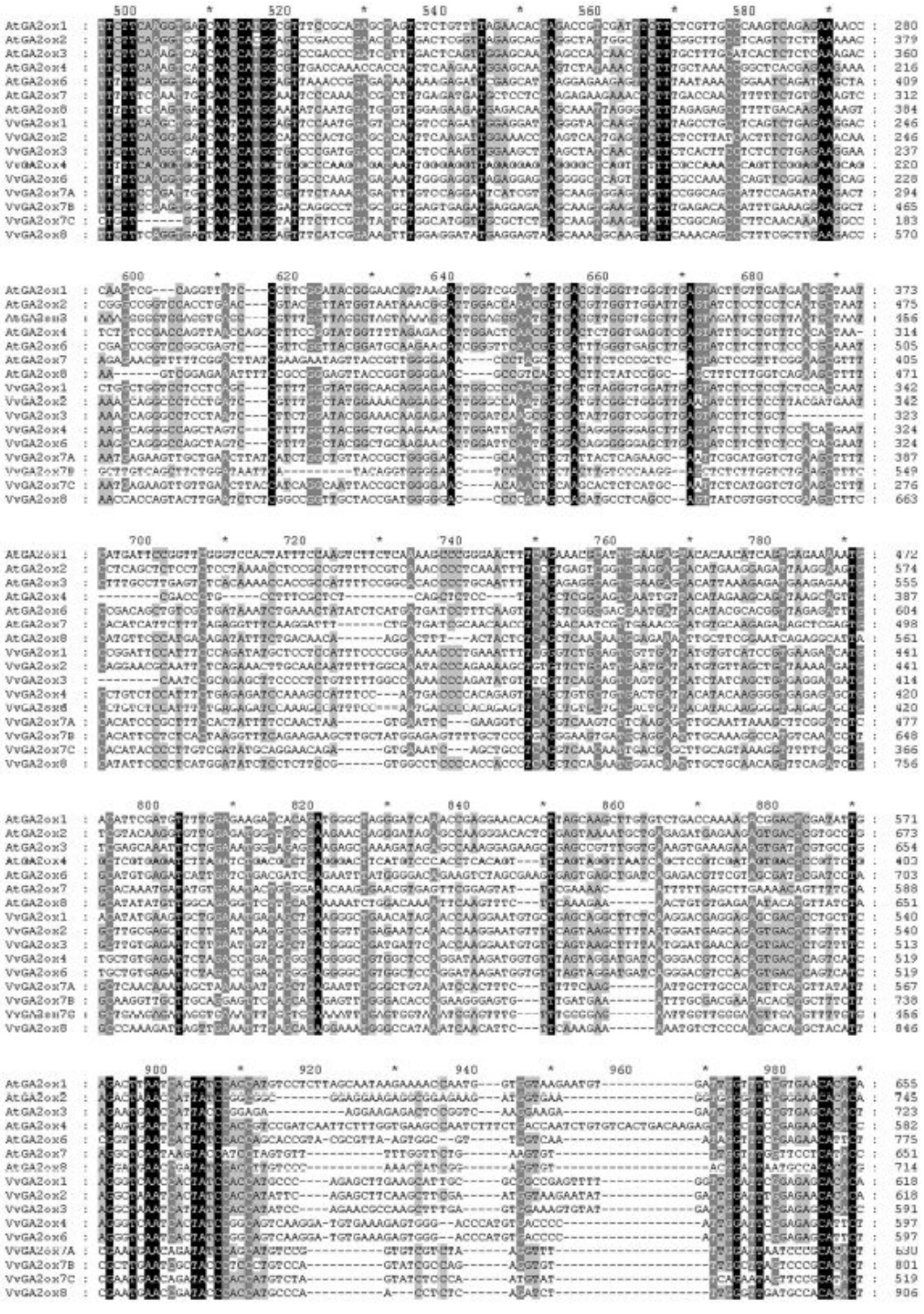
Supplementary Fig. 1-6. Comparative nucleotide sequence analysis of *Vitis* and *Arabidopsis* GA biosynthetic genes. Comparison of *VvGA20oxs* and *AtGA20oxs*.

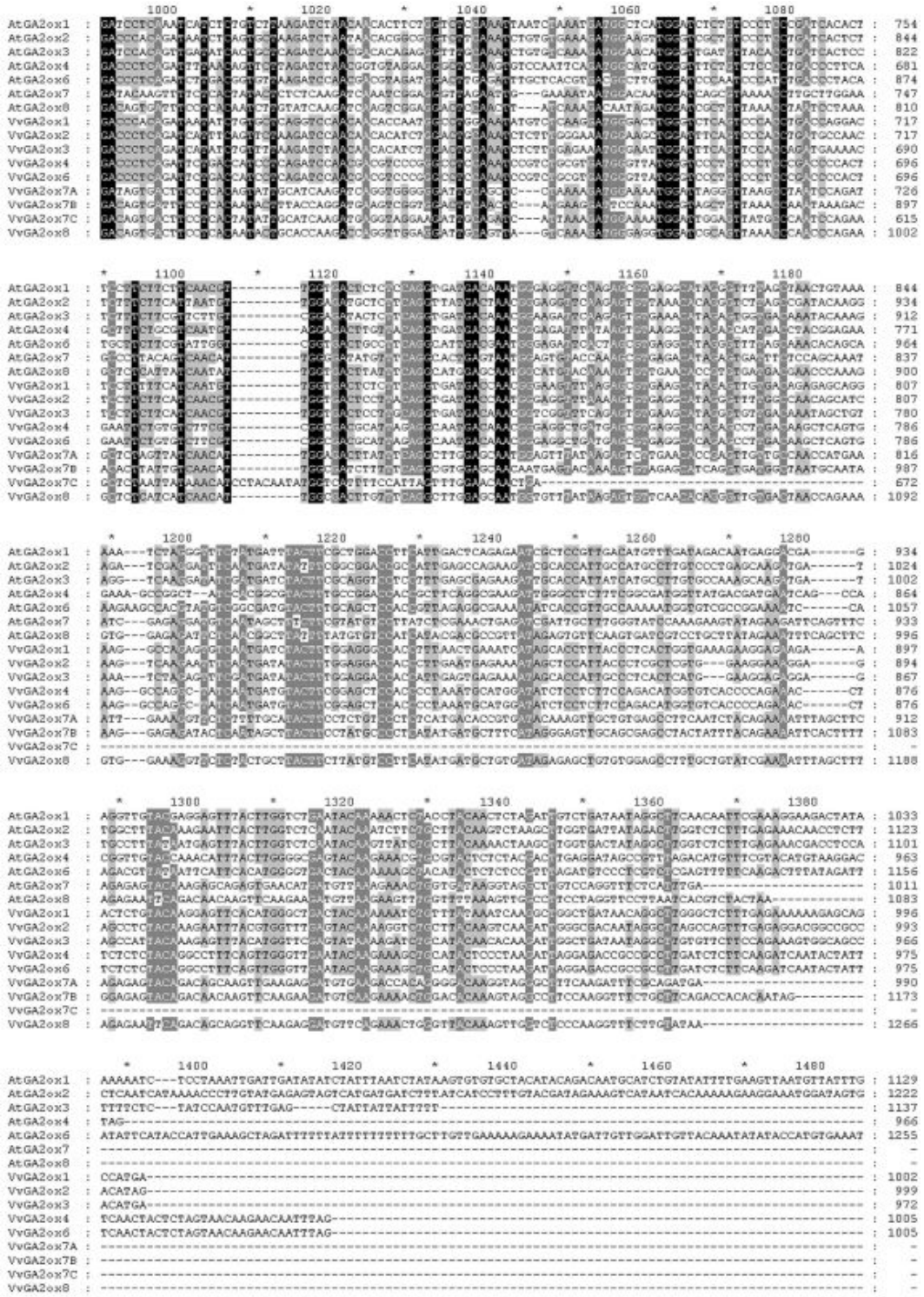


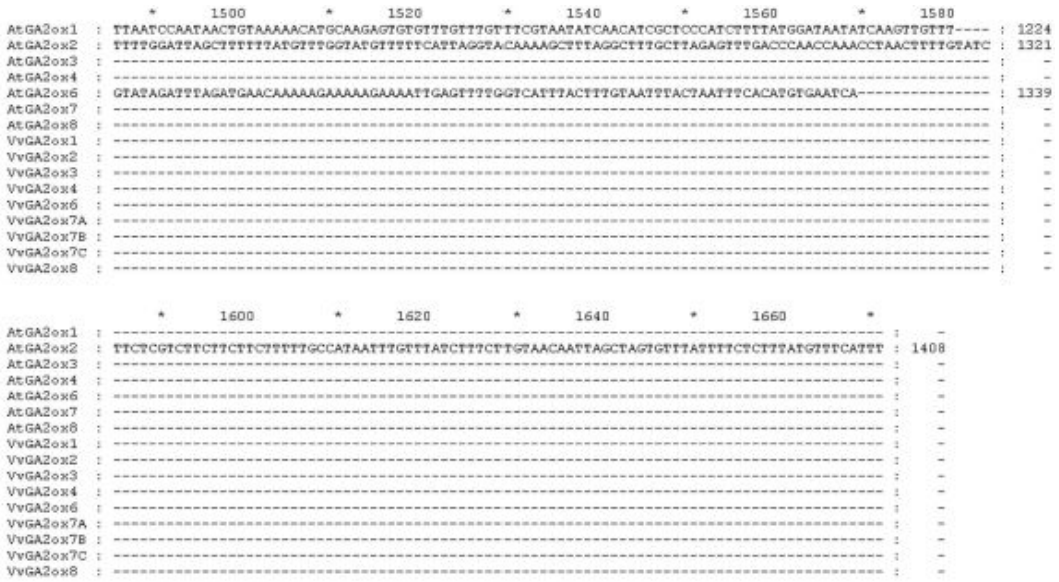


Supplementary Fig. 1-7. Comparative nucleotide sequence analysis of *Vitis* and *Arabidopsis* GA biosynthesis genes. Comparison of *VvGA3oxs* and *AtGA3oxs*.

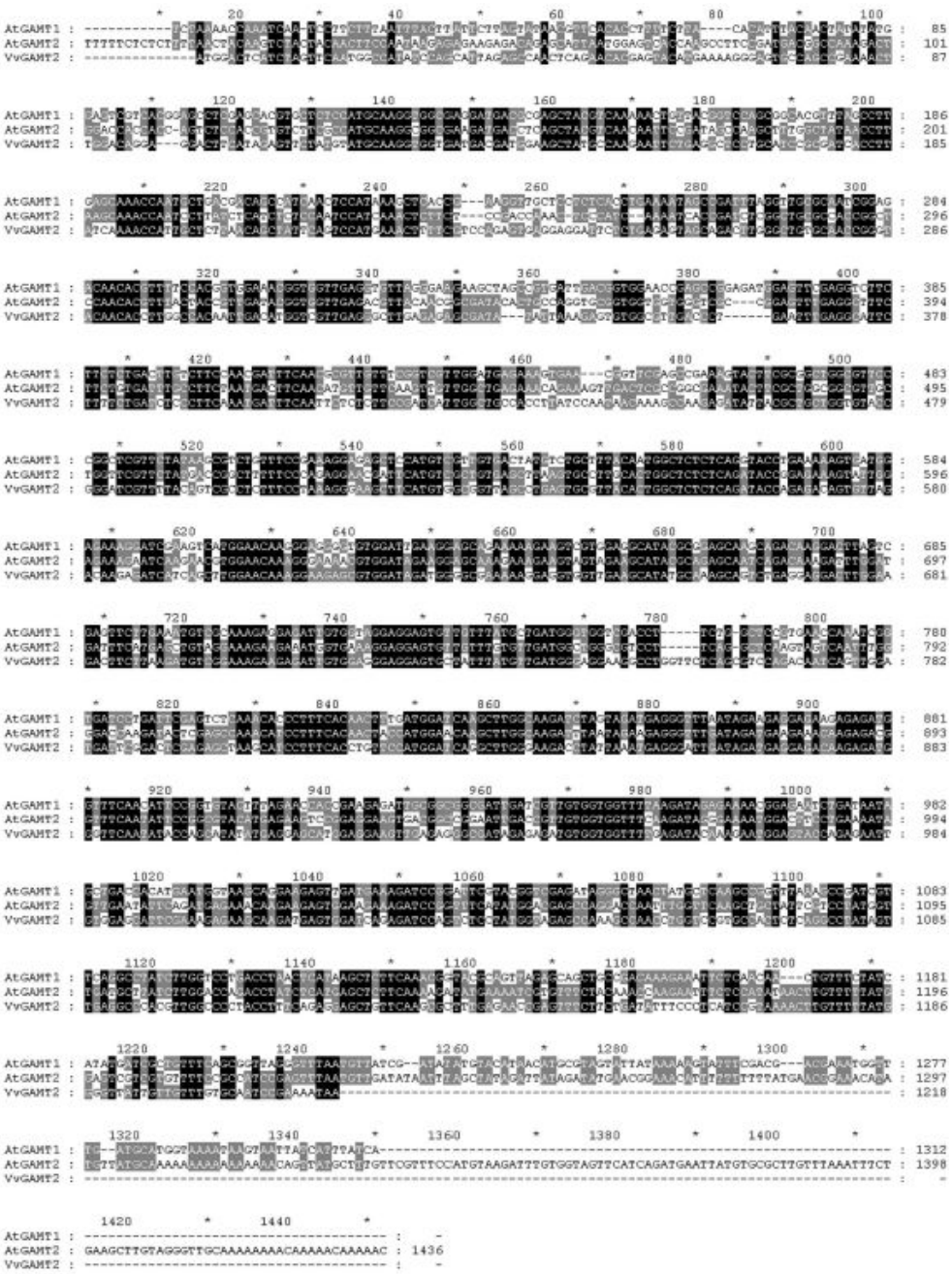




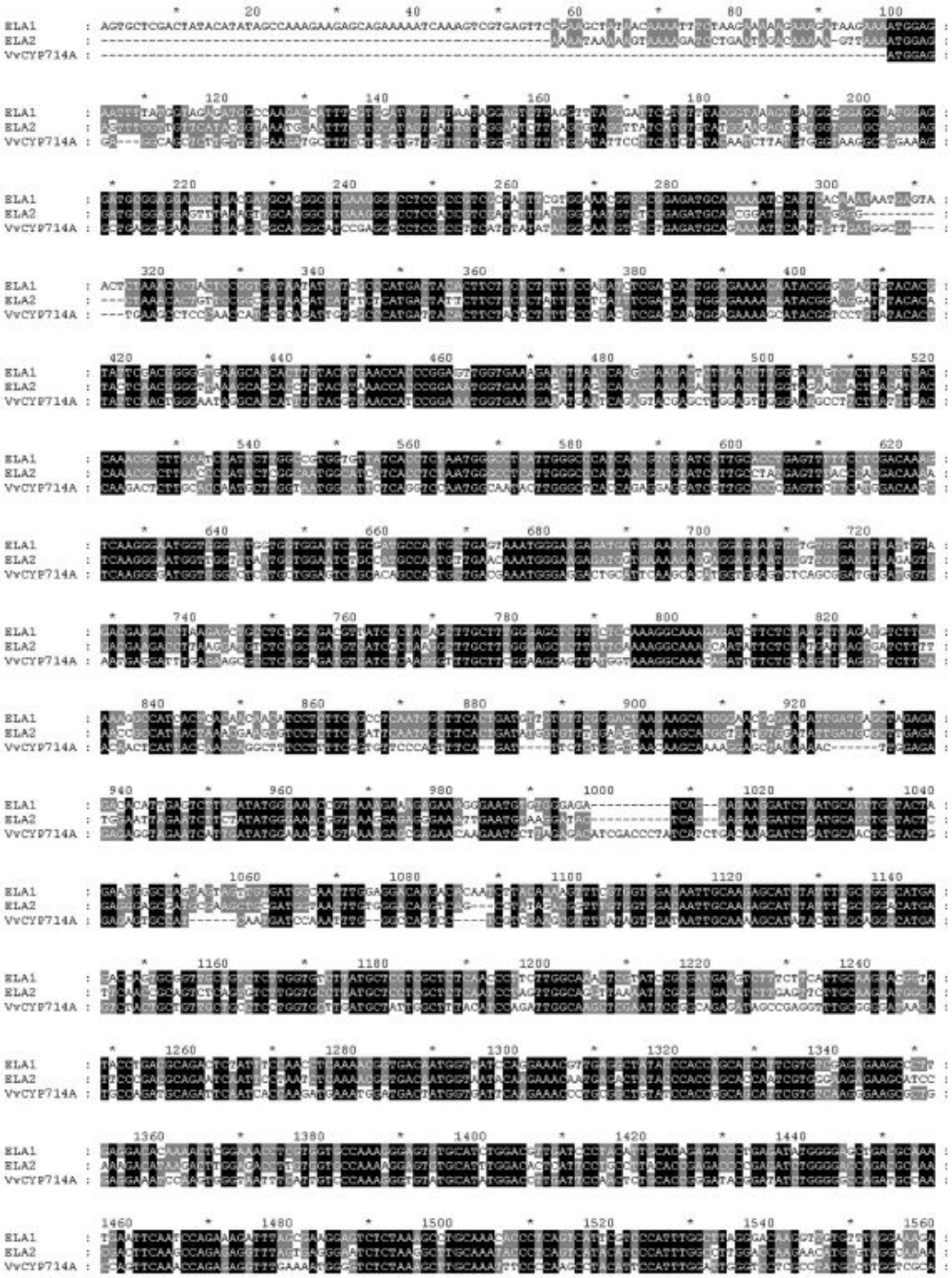


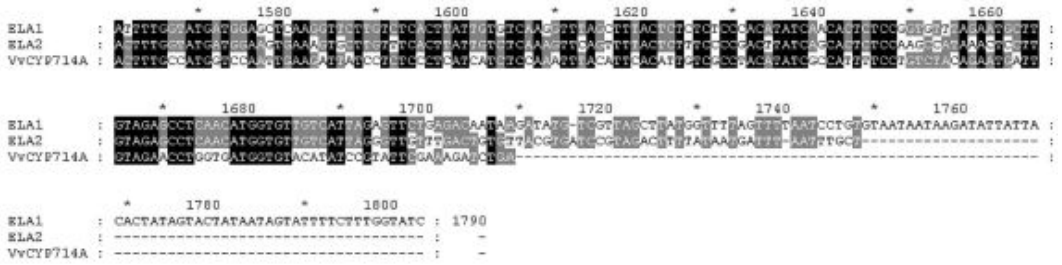


Supplementary Fig. 1-8. Comparative nucleotide sequence analysis of *Vitis* and *Arabidopsis* GA catabolic genes. Comparison of *VvGA2oxs* and *AtGA2oxs*.



Supplementary Fig. 1-9. Comparative nucleotide sequence analysis of *Vitis* and *Arabidopsis* GA catabolic genes. Comparison of *VvGAMT2* with *AtGAMT1* and *AtGAMT2*.


ELA1
ELA2
VvCYP714A



Supplementary Fig. 1-10. Comparative nucleotide sequence analysis of *Vitis* and *Arabidopsis* GA catabolic genes. Comparison of *VvCYP714A*, *ELA1*, and *ELA2.*

Supplementary Table 2-1. TIGR, SGN, or GenBank accession numbers of the proteins used for the phylogenetic analysis.

Protein family	Species	Protein name	Accession No.
ARF	*Arabidopsis thaliana*	AtARF1	NP_176184
		AtARF2	NP_851244
		AtARF3	NP_180942
		AtARF4	NP_200853
		AtARF5	NP_173414
		AtARF6	NP_174323
		AtARF7	NP_851047
		AtARF8	NP_198518
		AtARF9	NP_194129
		AtARF10	NP_180402
		AtARF11	NP_182176
		AtARF12	NP_174691
		AtARF13	NP_174679
		AtARF14	NP_174786
		AtARF15	NP_174784
		AtARF16	NP_567841
		AtARF17	NP_565161
		AtARF18	NP_567119
		AtARF19	NP_173356
		AtARF20	NP_174758
		AtARF21	NP_174701
		AtARF22	NP_174699

Supplementary Table 2-1. Continued.

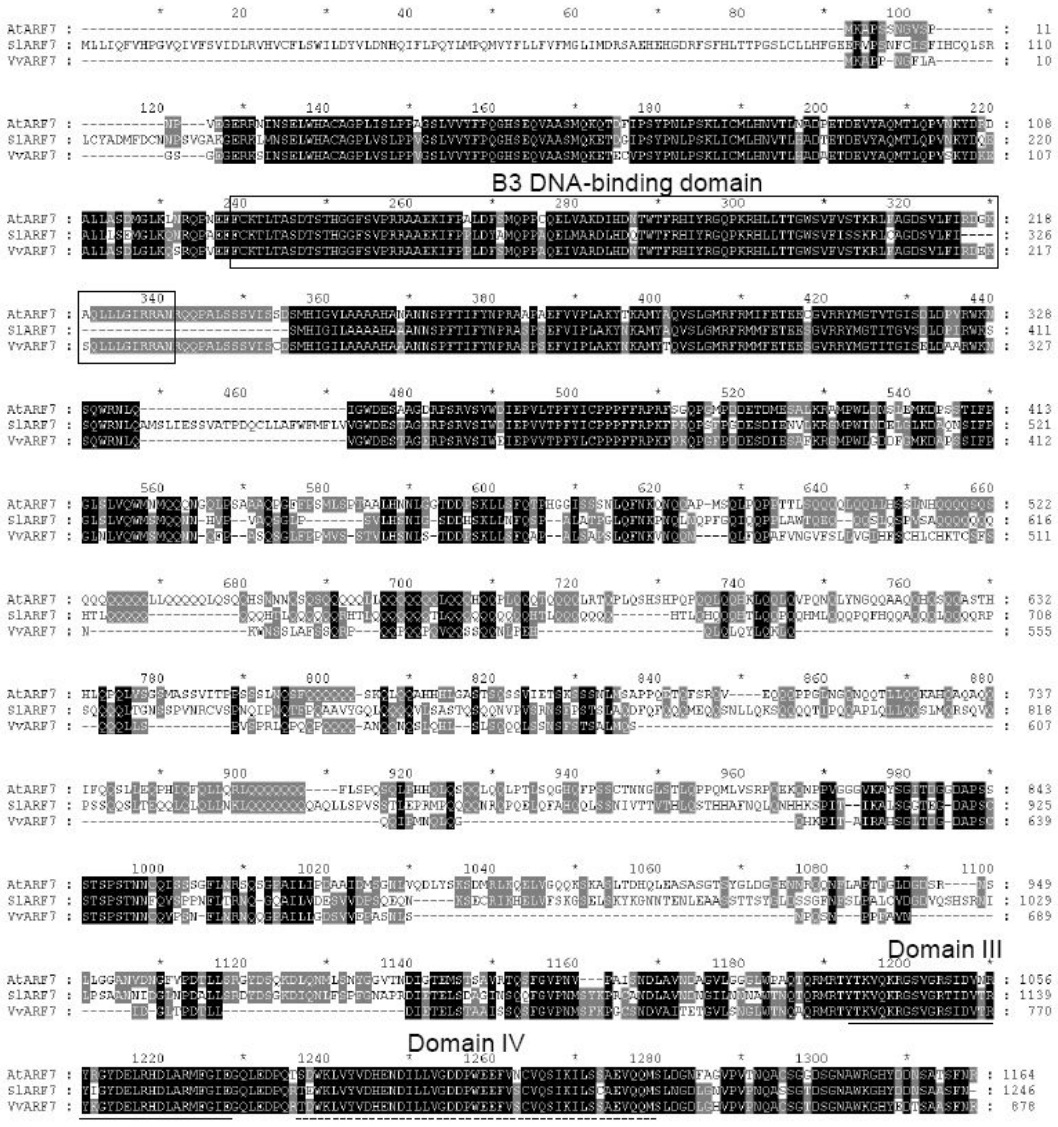
Protein family	Species	Protein name	Accession No.
ARF	*Solanum lycopersicum*	SlARF1	TC199727
		SlARF2	SGN-340284
		SlARF3	TC19246
		SlARF8*	TC198583
		SlARF5	TC198727
		SlARF6	SGN-U579438
		SlARF7	TC267250
		SlARF8	TC214335
		SlARF7*	EF121545
		SlARF10	ACU30063
		SlARF11	TC214573
		SlARF12	TC193011
		SlARF13	TC206384
		SlARF14	TC208143
		SlARF15	TC213824
		SlARF16	SGN-319318
	Vitis vinifera	VvARF7	CBI27770
		VvARF8	XP_002266678
YUC	*Arabidopsis thaliana*	AtYUC1	NP_194980
		AtYUC2	NP_193062
		AtYUC3	NP_171955
		AtYUC4	NP_196693
		AtYUC4s	NP_850808

Supplementary Table 2-1. Continued.

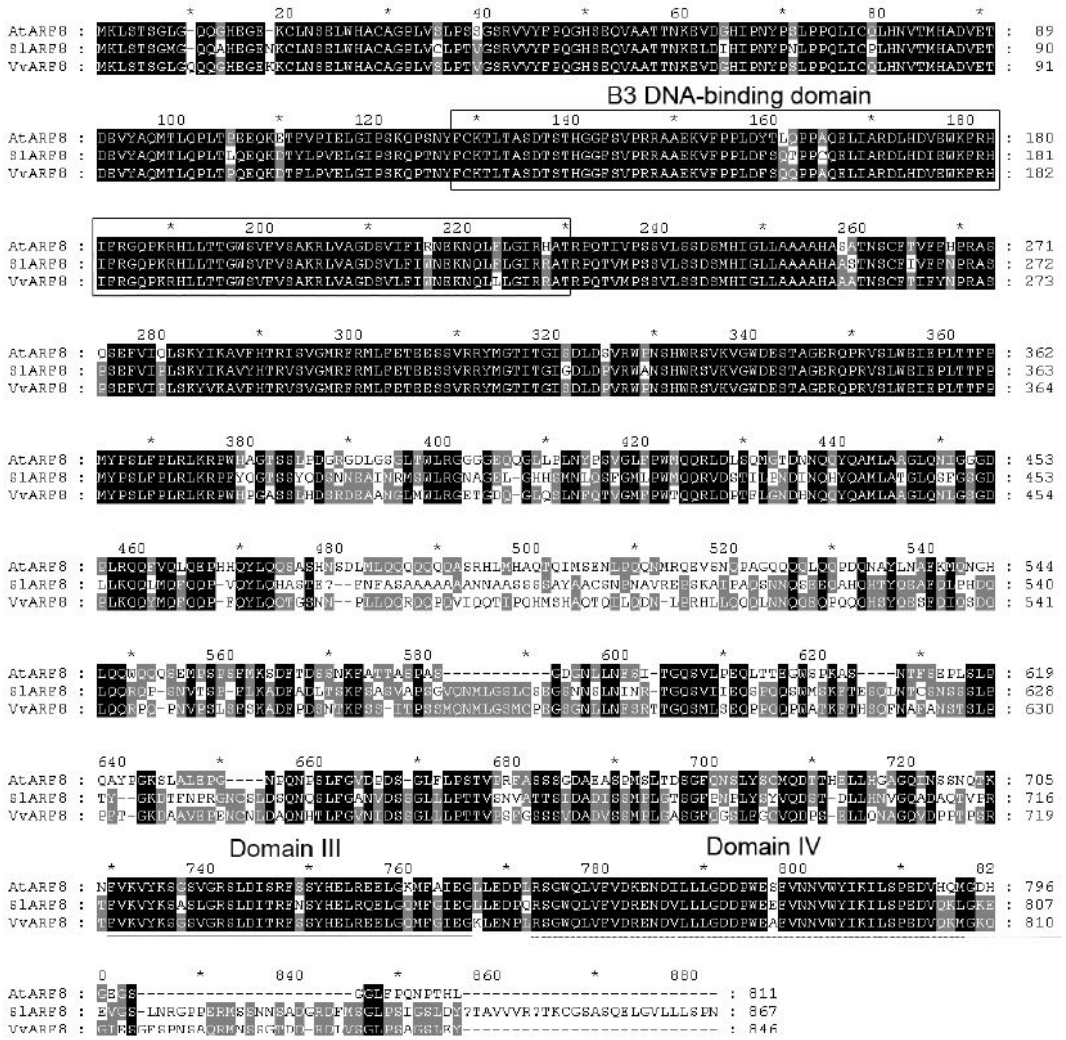
Protein family	Species	Protein name	Accession No.
YUC	*Arabidopsis thaliana*	AtYUC5	NP_199202
		AtYUC6	NP_001190399
		AtYUC7	NP_180881
		AtYUC8	NP_194601
		AtYUC9	NP_171914
		AtYUC10	NP_175321
		AtYUC11	NP_173564
	Solanum lycopersicum	ToFZY1	NP_001234343
		ToFZY2	XP_004245254
		ToFZY3	XP_004247940
		ToFZY4	XP_004240470
		ToFZY5	XP_004242376
		ToFZY6	XP_004247468
	Vitis vinifera	VvYUC2	XP_002281597
		VvYUC6	XP_002281015
DELLA	*Arabidopsis thaliana*	AtGAI	NP_172945
		AtRGA	NP_178266
		AtRGL1	NP_176809
		AtRGL2	NP_186995
		AtRGL3	NP_197251
	Solanum lycopersicum	SlDELLA	NP_001234365
	Vitis vinifera	VvDELLA	XP_002266267
		VvGAI1	XP_002284648

Supplementary Table 2-2. Primers used for qRT-PCR.

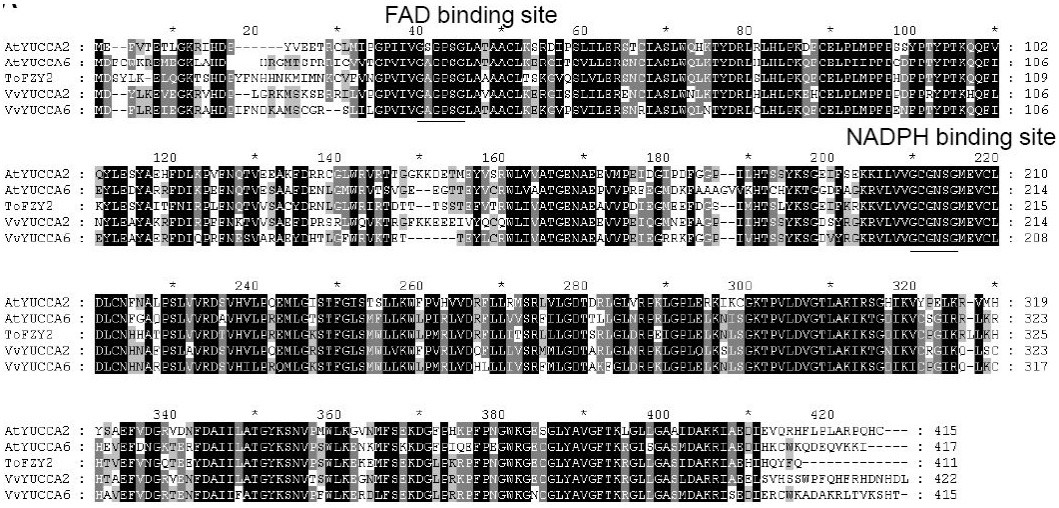
Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
VvARF7	CGTGCCTGTCCCAAATCAAGCT	CATCGATTAAATGAGGCAGCTGAGG
VvARF8	GGGGATCCTCTGAAACAGCAATACAT	AATGATTAATGGATTATTGCTGCCCG
VvIAA9	GGTGCCCAAACCGGTGAGTTAC	GAGCAAAAAAATTTATAACTCCAAACCTTCT
VvYUC2	CCCGGAGAATTTCCCGACTTACC	CCTTGGCTGTATATCGAACCGCTC
VvYUC6	GTGAGCGCGGAATTCGATCCT	TCCTCCTTTTTGAATCCCCGAGTC
VvDELLA	TCCCTCCTCTGCTTTCGCTTCC	GAGGACTCGGAGGGAGCAAGGA
VvGAI1	CACTCCCAATTGCGCCCTTG	CTGCAATTGGTGTAATCAAGAGGGG
VvActin1	CCCTCCTCTGCTTTCGCTTCC	GAGGACTCGGAGGGAGCAAGGA



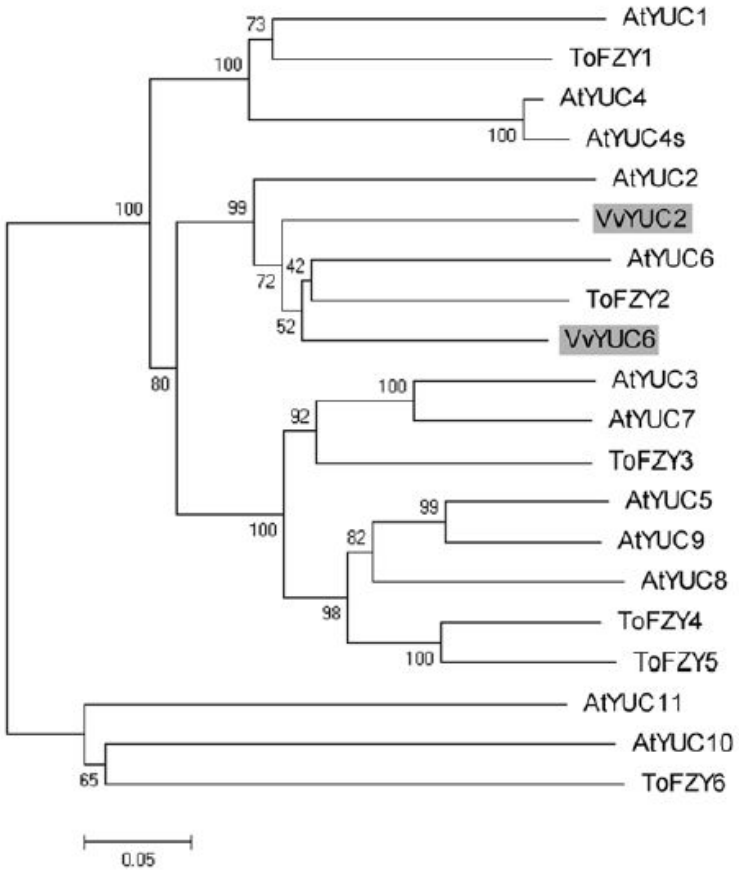
Supplementary Fig. 2-1. Protein sequence alignment of VvARF7. Comparison of the AtARF7, SlARF7, and VvARF7 amino acid sequences. The B3 DNA-*binding* domain is denoted with an open box. The Aux/IAA dimerization domains III and IV are underlined with solid and dashed lines, respectively. Identical and similar amino acids are shaded in black and gray, respectively.



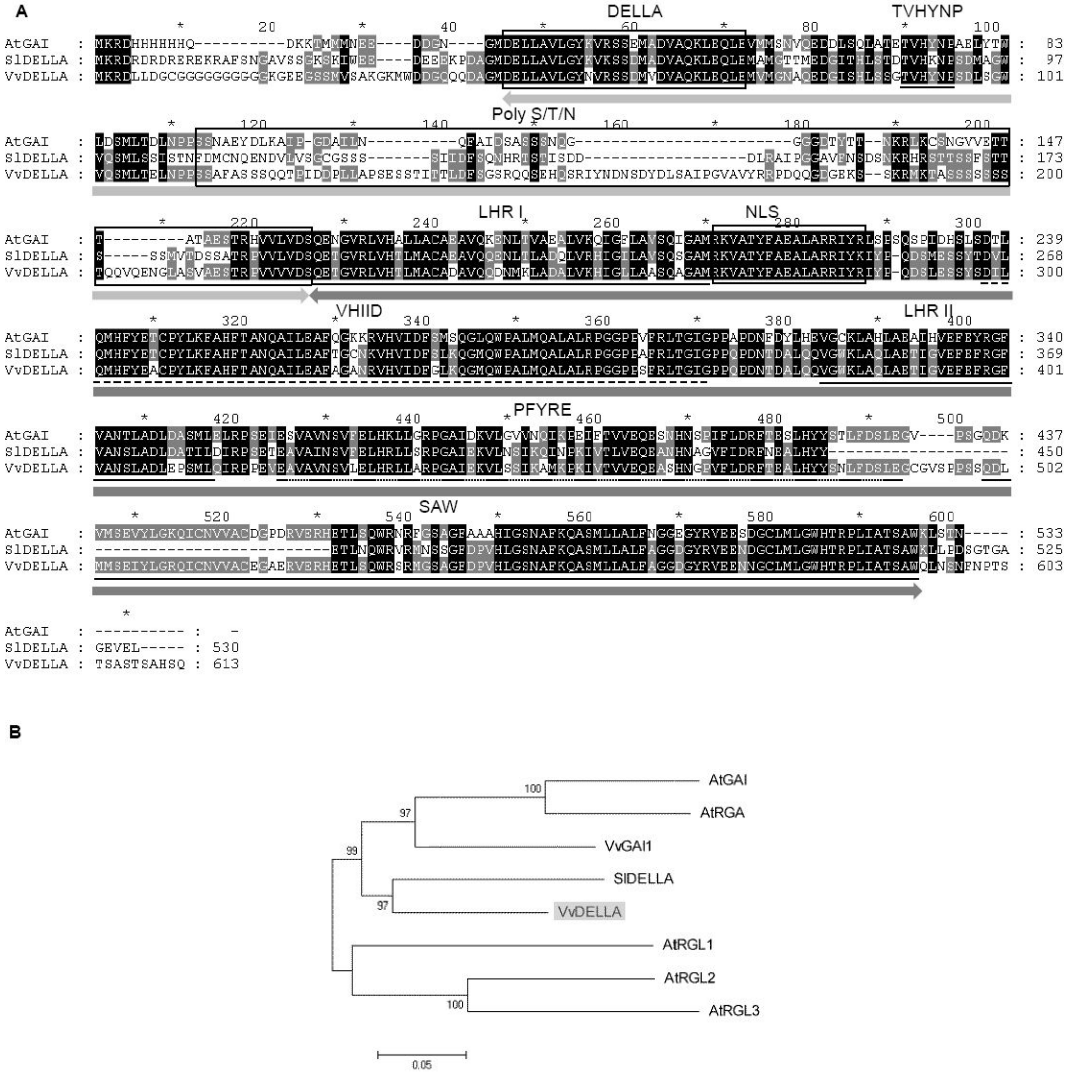
Supplementary Fig. 2-2. Protein sequence alignment of VvARF8. Comparison of the AtARF8, SlARF8, and VvARF8 amino acid sequences. The B3 DNA-*binding* domain is denoted with an open box. The Aux/IAA dimerization domains III and IV are underlined with solid and dashed lines, respectively. Identical and similar amino acids are shaded in black and gray, respectively.



Supplementary Fig. 2-3. Protein sequence alignment of VvYUC2 and VvYUC6. Comparison of the AtYUC2, AtYUC6, ToFZY2, VvYUC2, and VvYUC6 amino acid sequences. The FAD and NADPH binding sites are underlined. Identical and similar amino acids are shaded in black and gray, respectively.



Supplementary Fig. 2-4. A phylogenetic tree comparing VvYUC2 and VvYUC6 with *Arabidopsis* and tomato YUCCA families.


A
DELLA
TVHYNP
Poly S/T/N
LHR I
NLS
VHIID
LHR II
PFYRE
SAW
AtGAI : 83
SlDELLA : 97
VvDELLA : 101
AtGAI : 147
SlDELLA : 173
VvDELLA : 200
AtGAI : 239
SlDELLA : 268
VvDELLA : 300
AtGAI : 340
SlDELLA : 369
VvDELLA : 401
AtGAI : 437
SlDELLA : 450
VvDELLA : 502
AtGAI : 533
SlDELLA : 525
VvDELLA : 603
AtGAI : ---------- : -
SlDELLA : GEVEL----- : 530
VvDELLA : TSASTSAHSQ : 613
B
AtGAI
AtRGA
VvGAI1
SlDELLA
VvDELLA
AtRGL1
AtRGL2
AtRGL3
100
97
99
97
100
0.05
C
SlDELLA :
VvDELLA :
D
AtGAI :
AtRGA :
VvGAI1 :

Supplementary Fig. 2-5. Protein sequence alignment and phylogenetic analysis of VvDELLA. (A) Comparison of the AtGAI, SlDELL, and VvDELLA amino acid sequences. The DELLA and TVHYNP motifs for GA signal perception, the poly S/T/N motif for regulation of DELLA expression and the NLS motif for nuclear localization are denoted with open boxes. The LHR and VHIID domains for dimerization of DELLA protein are indicated with solid and dashed lines, respectively. The PFYRE and SAW domains that interact with the GA receptor are denoted with dotted lines. The DELLA and GRAS domains are indicated with light and dark gray arrows, respectively. Identical and similar amino acids are shaded in black and gray, respectively. (B) A phylogenetic tree of VvDELLA with five *Arabidopsis* DELLAs and one SlDELLA. Comparison of the Poly S/T/N motifs (C) between VvDELLA with SlDELLA and (D) between VvGAI1 with AtGAI.