VvHDZ28 positively regulate salicylic acid biosynthesis during seed abortion in Thompson Seedless

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Introduction

Seedlessness in grapes is one of the features most appreciated by consumers. However, the mechanisms underlying seedlessness in grapes remain obscure. Here, we observe small globular embryos and globular embryos in Pinot Noir and Thompson Seedless from 20 to 30 days after flowering (DAF). From 40 to 50 DAF, we observe torpedo embryos and cotyledon embryos in Pinot Noir but aborted embryos and endosperm in Thompson Seedless. Thus, RNA-Seq analyses of seeds at these stages from Thompson Seedless and Pinot Noir were performed. A total of 6442 differentially expressed genes were identified. Among these, genes involved in SA biosynthesis, VvEDS1 and VvSARD1, were more highly expressed in Thompson Seedless than in Pinot Noir. Moreover, the content of endogenous SA is at least five times higher in Thompson Seedless than in Pinot Noir. Increased trimethylation of H3K27 of VvEDS1 and VvSARD1 may be correlated with lower SA content in Pinot Noir. We also demonstrate that VvHDZ28 positively regulates the expression of VvEDS1. Moreover, over-expression of VvHDZ28 results in seedless fruit and increased SA contents in Solanum lycopersicum. Our results reveal the potential role of SA and feedback regulation of VvHDZ28 in seedless grapes.

Summary

Seedlessness in grapes is one of the features most appreciated by consumers. However, the mechanisms underlying seedlessness in grapes remain obscure. Here, we observe small globular embryos and globular embryos in Pinot Noir and Thompson Seedless from 20 to 30 days after flowering (DAF). From 40 to 50 DAF, we observe torpedo embryos and cotyledon embryos in Pinot Noir but aborted embryos and endosperm in Thompson Seedless. Thus, RNA-Seq analyses of seeds at these stages from Thompson Seedless and Pinot Noir were performed. A total of 6442 differentially expressed genes were identified. Among these, genes involved in SA biosynthesis, VvEDS1 and VvSARD1, were more highly expressed in Thompson Seedless than in Pinot Noir. Moreover, the content of endogenous SA is at least five times higher in Thompson Seedless than in Pinot Noir. Increased trimethylation of H3K27 of VvEDS1 and VvSARD1 may be correlated with lower SA content in Pinot Noir. We also demonstrate that VvHDZ28 positively regulates the expression of VvEDS1. Moreover, over-expression of VvHDZ28 results in seedless fruit and increased SA contents in Solanum lycopersicum. Our results reveal the potential role of SA and feedback regulation of VvHDZ28 in seedless grapes.
Salicylic acid promotes seedless in grape

genes responsible seedlessness (Nwafor et al., 2014; Royo et al., 2016; Xing et al., 2016), and research revealed that HD-Zip transcription factors play important roles in seed abortion in stenospermocarpic grapes, including VvHDZ28 and VvHDZ27 (Li et al., 2017). Nevertheless, our understanding of the seed abortion mechanisms in grapes remains rudimentary.

Here, seeds of Thompson Seedless (TS, stenospermocarpic, also known as Sultana) and of Pinot Noir (PN, seeded) were collected at 20, 30, 40 and 50 DAF. Morphological analyses of their seeds were carried out to determine the developmental differences between TS and PN. Also, endogenous hormones were quantified to determine the potential role of SA in seed abortion and RNA-Seq was carried out to identify the DEGs and candidate crosstalk genes. The relationship between VvHDZ28 and SA is predicted, as the expression of VvHDZ28 increases as seeds develop (Figure 1Cd) with the embryo sacs containing adaptor sequences were cleaned; clean reads were classified into four categories (0–1, 1–10, 1–100 and >100) (Figure S1c). Reads with CPM <1 were filtered and ignored in subsequent analyses (Figure S1d and Table S6). Coding genes and transcription factor were detected in each library (Figure S1e).

Principal component analysis (PCA) was used to assess the biological variability among all samples. The global transcript profiles of PN and TS samples were clearly distinguishable in each period, demonstrating the different gene expressions during seed development in these seeded and seedless grapes (Figure S1f). Strong correlations ($R^2 > 0.8$) in 20 selected genes were obtained between the results obtained using qRT-PCR and RNA-Seq (Figure S1g). This demonstrates the reliability of the data. Similar results was generated from scatter plots comparing the two years of data for PN and TS (Figure S4, Table S7). A total of 1129 transcription factors which can grouped into 39 transcription factor families were detected in seeds of PN and TS (Figure S1h and Table S5). They were expressed in PN and TS at varying levels.

**Results**

**Embryo aborts at 30 to 40 DAF in Thompson Seedless**

To study the seed development differences between seedless and seeded grapes, seeds were collected from TS and PN at 20, 30, 40 and 50 DAF in 2013 and 2014 (Figure 1A). In PN, longitudinal diameter, transverse diameter and fresh weight of seeds and berries all increased as berries developed. In TS, for berries, the longitudinal diameter, transverse diameter and fresh weights all increased from 20 to 50 DAF but for seeds those indexes increased from 20 to 30 DAF but then decreased from 30 to 50 DAF (Figure 1B). The fresh weights of berries of PN and TS were similar but the seeds of PN were heavier than those of TS. Seeds shrinkage was significant by 40 and 50 DAF, compared with at 30 DAF. This result indicates embryo abortion during 30–40 DAF in TS.

To further investigate differences in seed development between PN and TS, the internal morphogenesis of seeds for each period was analysed using paraffin sections. In PN, the embryo developed to a small globular embryo (Figure 1Ca), a globular embryo (Figure 1Cb), a torpedo embryo (Figure 1Cc) and to a cotyledon embryo (Figure 1Cd) with the embryo sacs filled with endosperm on 20, 30, 40 and 50 DAF, respectively. In TS, the embryo developed to a small globular embryo (Figure 1Ce) and a globular embryo (Figure 1Cf) on 20 and 30 DAF, respectively; however, the embryo disappeared and the endosperm began to abort on 40 DAF (Figure 1Cg), and the embryo sac was empty on 50 DAF (Figure 1Ch). This pattern indicates that in (the Yangling region) the embryo and endosperm of TS develop normally in the early stages but abort at about 30–40 DAF.

**Transcriptome sequencing and assembly**

In TS, the embryo and endosperm of seeds develop normally from 20 to 30 DAF, but abort at about 40 or 50 DAF. This indicates something happens between about 20–50 DAF. The study then focused on this period to gain greater insight into the mechanism of embryo abortion. Libraries of PN and TS seeds from 20, 30, 40 and 50 DAF were constructed and sequenced. Transcript reads containing adaptor sequences were cleaned; clean reads were mapped to the *Vitis vinifera* L. genome Ensembl v26 (Figure S1a). An average of 75.76% of these reads was uniquely mapped to a single genomic locus and 2.97% was mapped to multiple locations in the whole genome (Table S2). To make the gene transcript level more precise, the gene boundary was revised before analysis (Tables S3, S4 and Figures S2 and S3). Totals of 35784 genes (PN) and 32286 genes (TS) were obtained (Figure S1b and Table S5). Based on counts per million (CPM), the gene expression levels in PN and TS transcriptomes were classified into four categories (0–1, 1–10, 1–100 and >100) (Figure S1c). Reads with CPM <1 were filtered and ignored in subsequent analyses (Figure S1d and Table S6). Coding genes and transcription factor were detected in each library (Figure S1e).

**Identification of differentially expressed genes in Thompson Seedless and Pinot Noir**

Differentially expressed genes were identified in two stages for PN and TS (Figure 2Aa,b). A two-class time course analysis to directly compare the developmental series between TS and PN was carried out, differential expressed genes were identified (Table S8 and S10), and GO enrichment of DEGs was performed (Table S9). However, no DEGs could be detected on 50 DAF vs on 40 DAF in PN, possibly due to the maturation of the seeds. The number of DEGs increased as seeds developed (Figure 2Aa,b).

Of the transcription factors detected in seeds of PN and TS, most members of the HD-Zip and GRAS families of transcriptional regulators were highly expressed in TS at 20 and 30 DAF, whereas they had low expressions in PN (Table S5). Genes differentially expressed only in PN participate in seed development, embryo-genesis and hormone, while genes differentially expressed only in TS participate in seed development, hormone and lignin synthesis. Genes related to hormones, seed development and embryo development were detected in both PN and TS (Figure 2D).

**Functional enrichment of differentially expressed genes in Thompson Seedless and Pinot Noir**

GO enrichment analysis was carried out on DEG gene clusters, and enrichment map (EM) was constructed by using enriched GO categories with FDR-adjusted $P$ value < 0.01 (Ma et al., 2014; Merico et al., 2010) to visualize GO categories as a network. The GO categories with parent–daughter relationships in EM were clustered into modules, which were connected by genes shared between the GO categories.

A total of 6442 DEGs can be classified into eight clusters by GO category (Figure S5a, Table S11 and S12). The ‘development and growth’ category contained a large fraction of genes that were specifically involved in post-embryonic development (GO:0009886) and post-embryonic organ morphogenesis (GO:0048563). The cell-wall-organization genes mostly encode proteins involved in secondary cell wall biogenesis (GO:0009834) and specification of axis polarity (GO:0065001). These genes may not be directly involved in seed development but could be associated with one
Hormone co-express genes during seeds development

Hormones have been shown to play important roles in plant embryogenesis (Finkelstein et al., 2002; Liu et al., 1993; Marion-Poll, 1997). Endogenous hormones were quantified during seed development in PN and TS, in which SA showed significant differences between the two genotypes, being more concentrated in the seeds of TS than in those of PN at all four stages measured (Figure 3A). Meanwhile, GA and BR showed opposite-going expression patterns in PN and TS. Contents of ABA were decreased at 20–50 DAF in both PN and TS, but higher contents were detected in TS than PN except DAF30. Dose of JA was decreased in TS while opposite trends were founded in PN at 20–30 DAF. IAA showed opposite expression pattern with JA in PN and TS. ACC has no obvious difference in PN and TS except lower concentration in DAF50 in PN.

Figure 1 Seeds and morphological analyses of samples taken 20, 30, 40 and 50 days after flowering (DAF) from Pinot Noir and Thompson Seedless. (A) Seeds and berries of 20, 30, 40 and 50 DAF from Pinot Noir and Thompson Seedless. (B) Longitudinal diameters, transverse diameters and fresh weights of seeds, 30 berries were used for data analysis. (C) Morphological analyses of seeds. a-d, small globular embryo, globular embryo, torpedo embryo and cotyledon embryo for seeds at 20, 30, 40 and 50 DAF of Pinot Noir, e-g, small globular embryos, globular embryos, embryo has disappeared and endosperm begins to disintegrate, endosperm disintegrated and seed coat left for seeds at 20, 30, 40 and 50 DAF from Thompson Seedless. Embryo location is denote by red arrow. Bar: a, b = 5 μm, c, d = 100 μm, g, h = 1 mm.
Hormone interactions regulate meristem function and control organ formation (Vanstraelen and Benkova, 2012; Nordstrom et al., 2004). Strong positive correlations among BR, GAs, CTK and ET occurred in both two cultivars (Figure 3B and Table S12). To gain further insight into the potential relationship between DEGs and embryo development-abortion, co-expression analyses were carried out in PN and TS, based on the mRNA and hormone data to identify DEGs likely to be co-regulated with differentially expressed hormones. A total of 369 DEGs were co-expressed with at least two hormones in both PN and TS. Also, a large number of genes were co-expressed with ABA, SA and IAA (Figure 3C, Table S13 and S14).

**ABA2** (VIT_08s0007g02700) encodes a short-chain dehydrogenase/reductase involved in the conversion of xanthoxin to ABA-aldehyde during ABA biosynthesis (Lin et al., 2006). It was found to correlate with ABA, tZ and tZR, and shows a similar expression pattern to ABA, tZ and tZR accumulation. Meanwhile, **AHP4** (VIT_09s0002g03520) is a positive regulator of cytokinin signalling (Hutchison et al., 2006), and this has similar expression pattern to IAA content. In contrast to PN, most of these genes had relatively high expressions in TS at 20 DAF (Figure 3D).

**SA biosynthesis-related genes and cell death genes have higher transcript level in TS**

The **EDS1** and **SARD1** genes play pivotal roles in regulating SA biosynthesis in plants (Dempsey et al., 2011; Jirage et al., 1999; Wang et al., 2011). The homologous genes to **EDS1** and **SARD1** showed higher transcript levels in TS than in PN (Figure 4a). The SA marker genes, including **PR1** and **PR5**, also expressed more highly in TS than in PN. This is consistent with the SA concentrations in the seeds of PN and TS (Figure 4a,b), similar situation of SA concentration and SA related genes expression was founded in vegetable organs of PN and TS (Figure S7). Salicylic acid has long been studied for its role in response to pathogen attack and for its induction, and tightly associated with PCD. Here arises the question whether the accumulation of SA in seeds of seedless grape is functionally related to embryo abortion or merely correlated with it? We also found that genes downstream of SA, **NPR1**, γVEP and **WRKY6** had higher expression levels in the seeds of TS than in the PN ones. We speculate that high levels of SA induce over-expression of the cell death genes causing embryo abortion in seedless grapes. **HDZ28**, which belongs to HD-ZIP gene family, has higher expression in four stages of TS than in PN (Figure 4b). Moreover, pHDZ28 was induced by SA (Figure 4c,d).

**H3K27me3 of SA biosynthesis genes evaluated in PN**

The similarity of the **EDS1** and **SARD1** promoter sequences is 99.27% and 99.57% in TS and PN, respectively (Figure S6a,b). A number of recent reports show that chromatin modifications play important roles during seed development (Grimanelli, 2012; Kawashima and Berger, 2014). This leads us to question whether chromatin modifications could be involved in seed develop/abortion in PN/TS. ChIP and qRT-PCR were used to examine histone modification of **EDS1** and **SARD1** in PN and TS. Chromatin was extracted from seeds of PN and TS using anti-H3AC and anti-H3K27me3 antibodies. For **EDS1** and **SARD1**, a region close to the translation initiation codon (ATG, region 1) and another exon region downstream of the ATG (control, region 2) were examined (Figure 5a,c). As predicted, there was a significant increase in H3K27me3 in the promoter regions of **EDS1** and **SARD1** in PN (Figure 5b,d). No significant differences in the levels of H3AC in region 1 and region 2 in PN and TS (Figure 5b,d). Expression of **HDZ28** was increased after SA
treatment while expression of SARD1 and EDS1 has no significant variations (Figure S8). These results indicated that lower expression of these two genes and SA concentration in the seeds of PN were generated from increased H3K27me3 in the promoter region.

VvHDZ28 positively regulates expression of EDS1

The protein in the HD-zip I family has been reported to recognize and bind to the TAATTA cis-element (Gao et al., 2016). In promoter of SARD1 and EDS1, 6 and 3 TAATTA and its similar sequence ATTAATT for VvHDZ28 were found (Figure S9a,b). Analysis of the infiltrated plants reveals that VvHDZ28 can activate pSARD1 and pEDS1 luciferase (LUC) reporter gene while fragment promoters containing no cis-elements are not affected (Figure S9c,d). GUS staining and assay of HDZ28+pSARD1 and HDZ28+pEDS1 was higher than pSARD1 and pEDS1 (Figure S9e-h).

To test whether VvHDZ28 has DNA binding specificity for the pSARD1 and pEDS1, the SARD1 and EDS1 promoter regions were cloned a LacZ reporter gene to form a reporter construct, and the VvHDZ28 coding sequence was fused with the yeast activation domain to form the effector AD-VvHDZ28 construct. Both the effector AD-VDHDZ28 (AD alone as a negative control) and the reporter constructs were cotransformed into yeast. As shown in Figure S10, VvHDZ28 could bind to the EDS1 promoter but not SARD1. These results suggest that this pEDS1 region contains VvHDZ28 binding sequences. Then, fragment promoters containing cis-elements were cloned in front of a LacZ reporter gene to form a reporter construct, and effector AD-VvHDZ28 construct cotransformed into yeast, the results revealed that P3 fragment containing cis-elements (TAATTA changed to TGGTTG) of EDS1 gene indeed abolished LacZ activation (Figure S10). Thus, we propose that VvHDZ28 positively regulates the expressions of EDS1 by binding to the promoter of it.

Endogenous SA concentrations increased in OE-HDZ28

Transgenic plants were generated with ectopic expression of HDZ28 in Micro-tom driven by a CaMV35S promoter to study its functional role in development of plant organ (Figure S11a). 18 (90%) independent plants were identified in the T1 generation.

Figure 3 Hormonal content of seed development in PN and TS and hormonal co-expressed gene analysis. (A) SA, salicylic acid, GA, gibberellin, ABA: abscisic acid, BR: brassinosteroids, IAA: auxin, CTK: cytokinin, JA: jasmonic acid, ACC: aminocyclopropane-1-carboxylic acid, precursor of ethylene. Capital letter represents comparison of PN and TS at each stage (P < 0.05); lowercase letters represent comparison among different stages of PN and TS (P < 0.05). (B) Hormonal correlation analysis. (C) Genes co-expressed with endogenous hormone in PN and TS. (D) Heat map of 463 genes co-expressed with at least two hormones.
by PCR amplification, in which OE-HDZ28 1–3, 1–6 and 2–3 were selected for further analysis. VvHDZ28 was detected in leaves, roots, flowers, shoots and fruits of transgenic plants (Figure S11d, e). Southern blot hybridization signal was detected in OE-HDZ28 plants using a 361 bp DNA probe but not in the WT control (Figure S11c, lane 1).

To assess whether OE-HDZ28 affects endogenous SA content and expression of EDS and SARD, we quantified endogenous SA concentrations and transcript levels of EDS and SARD during inflorescence development in OE-HDZ28 and WT plants. The SA content was also increased in OE-HDZ28 at all stages (Figure 6v). SARD and EDS paralogous genes in tomato had higher expressions in OE-HDZ28 (Figure 6w,x, Figure S12). The results further confirm the idea that VvHDZ28 positively regulates the expression of SARD and EDS, thus affecting SA concentration in TS.

**OE-HDZ28 caused abnormal flower development and parthenocarpic fruits**

At anthesis, WT tomato flowers usually have five green sepals, five yellow petals and five fused yellow stamens surrounding a gynoecium of five fused green carpels (Brukhin et al., 2003). The
OE-HDZ28 developed flowers with the first whorl usually having six fused sepals (Figure 6c), also fused carpels and stigmas. Swollen ovaries were also visible in OE plants (Figure 6f–h). However, the third whorl (stamens) was not always found in the OE-HDZ28 flowers; characterization of two more independent lines was shown in Figure S13. Under our growing conditions, parthenocarpy occurred in 10% of flowers and fruits developed in the OE-HDZ28 plants (Figure 6j). Thus, homozygous progeny of OE-HDZ28 cannot be obtained by self-pollination; T1 generation plants of OE-HDZ28 were used for functional analysis.

Histological sectioning of flower morphogenesis showed that the floral organ primordia initiated in a similar way in the OE-HDZ28 and WT plants (Figure S14a,b). As in the WT, four whorls were initially formed in OE-HDZ28, while fewer ovules and thicker carpels were also observed. Significant differences were visible later on during floral organ differentiation and development (Figure S14c–f), stamens disappeared as the flower developed (Figure S14g,h). Also, the number of ventricles increased in OE-HDZ28 plants but not in the WT ones. Moreover, in some ventricles, ovules were not formed in the OE-HDZ28 flowers (Figure S14h). Apparently, OE-HDZ28 does not affect vegetative development, flowering time or inflorescence architecture.

We investigated whether over-expression of VvHDZ28 in tomato affected floral organ identity genes by analysing the expressions of tomato-flower-related genes (Pan et al., 2010;
Pnueli et al., 1994; Quinet et al., 2014). MC and TM6 transcript levels were significantly higher in the OE-HDZ28 than in the WT plants, both in the flowers when young and at anthesis. The $SlTM4$ was not significantly affected in the OE-HDZ28 plants but $SlTAP3$ and $SlTAG1$ decreased in the flower buds (Figure S14i).
OE-HD228 exhibits plant pollen tube growth and fertility

Fruit set and seed development depend on successful pollen germination followed by successful pollen tube growth and fertilization. However, the OE-HD228 plants were without stamens. To determine whether pollen is able to germinate on OE-HD228 plants, these were pollinated with pollen from WT plants. By 24 h after pollination, pollen had germinated in both the OE-HD228 (Figure 6l,m) and the WT plants (Figure 6o,p). However, while pollen tubes reached the ovules in the WT plants (Figure 6m), only a few pollen tubes did so in the OE-HD228 plants (Figure 6p). In contrast with the WT plants, in the OE-HD228 plants, 10% of the pollinated flowers produced fruits without seeds (Figure 6s) and another 10% of flowers produced fruits with seeds in some ventricles (Figure 6t) but seed numbers were low. The remaining 80% of ovules did not develop but shrivelled (Figure 6l, Table S15). These results indicate that WT pollen can germinate on OE-HD228 flowers but fertilization happens only in a few flowers.

Discussion

Embryogenesis is a critical stage in higher plant reproductive cycles, it begins after a double-fertilization event, with embryo and endosperm forming thereafter (Capron et al., 2009; Harada, 1999). To determine the differences between seedless and seeded grapes, endogenous hormones were quantified and morphological analyses were carried out of seeds at 10-day intervals. Transcriptomic surveys were also carried out at four stages of seed development that included normal development and the abortion of the embryo and endosperm. Seed fresh weight also decreased as seed abortion initiated, this result is in accordance with previous reports (Wang et al., 2016). We found that fertilized seeds formed both in TS and in PN as expected. However, while viable seeds formed in the seeded grape, the embryo and endosperm stopped developing in TS and soon degenerated, to leave only a seed trace (Figure 1c).

Salicylic acid can induce pathogen defence responses associated with PCD (Brodersen et al., 2005; Radojićić et al., 2018; Rasmussen et al., 1991); it also regulates seed germination, flower formation and senescence (another type of cell death which is different from the hypersensitive response) (Martínez et al., 2004; Rajjou et al., 2006; Xie et al., 2007). SARD and EDS are both key regulator genes in SA biosynthesis (Nawrath et al., 2002; Wang et al., 2011). SA and its related genes also have potential roles in promoting seeds abortion in grapes (Royo et al., 2018). In our study, the VIT_07s0031g02390 gene, homologous to the EDS1 gene in Arabidopsis, had higher expression in TS than in PN. ChIP analysis reveals histone modification of SARD1 and EDS1 (H3K27me3) in PN, which seems to be the cause of the differential expression levels in PN and TS (Figure 5).

PR1 and PR5 have been reported to have higher expressions in response to SA evaluation. NPR1 is an important gene regulating expression of PR1 and PR5 and cell death in the SA signalling pathway (Cao et al., 1994; Nawrath and Métraux, 1999). All of these have higher expressions in TS than in PN. Moreover, yPVE is a positive regulator of PCD (Hara-Nishimura and Hattori, 2011; Kuroyanagi et al., 2005). Meanwhile, AtWRKY6 has been shown to be induced by SA and to participate in senescence and defence responses (Robatzek and Somssich, 2001). Both of these have higher transcript levels in TS than in PN. Based on this study, we suggest SA plays a positive role in seedless grapes. However, high levels of SA alone are not sufficient to activate cell death (Radojićić et al., 2018); there may have other factors that take part in PCD during seedless of grape. NB-LRRs work upstream of SA and may function as general cell death receptors that recognize a variety of pro-cell death signals in plant immune system (Bombilas et al., 2007; Caplan et al., 2008), cell death during seed development in TS could be due to activation of defence regulators genes encoding NB-LRR receptors, this need to be verified in the future.

6442 DEGs were identified in seed transcriptome of TS and PN, including genes that have been proved to have potential relationships with seed abortion in grape, such as ch-Cpn21, AGL11 and STS (Bouquet and Danglot, 1996; Hanania et al., 2007; Ingrosso et al., 2011); genes homologous to embryo abortion genes in other species, including WRKY2, WOX9 and metacaspase genes (Colette et al., 2015; Ueda et al., 2011; Zhang et al., 2013; Zhu et al., 2014); and transcription factor families also differentially expressed, for example HD-Zip, NAC and WRKY (Tables S5 and S8). Among these, the HD-Zip gene family has long been studied for its role in embryogenesis (Ariel et al., 2007; Colette et al., 2015; Li et al., 2019; Li et al., 2017; Ogawa et al., 2015; Prigge et al., 2005). A total of 21 of these were differentially expressed in PN and TS, and most had higher transcript levels in TS than in PN (e.g. VvHDZ228). We propose this gene family plays a pivotal role in seed abortion as 29 transcription factors of this family were found to be differentially expressed in seedless and seedled progeny of Red globe and Centennial Seedless (Wang et al., 2016).

VvHDZ28 is homologous to the ATHB12 transcription factor in Arabidopsis. It has been shown to suppress expression of GA20ox and to decrease GA biosynthesis (Son et al., 2010). Also, it is induced by ABA and by water deficit (Olsson et al., 2004; Ré et al., 2014; Son et al., 2010). SARD1 and EDS1 have binding sites for HD-ZIP I proteins (Figure 7a,b). Our results suggest VvHDZ28 positively regulates EDS1 transcript by binding to its promoter (Figure S9 and S10). Thus, SA content in TS and in OE-HD228 tomato plants was higher in contrast with PN and WT tomato.
plants, although expression pattern of VvHDZ28, VvSARD1 and VvEDS1 is different in each stage.

Seedlessness is a popular characteristic in table grape, so it is important to develop seedless transgenic grapes. HD-Zip transcription factors are predicted to play pivotal roles in the grape embryo abortion process, including VvHDZ27 and VvHDZ28 (Li et al., 2017). OE-HDZ28 plants lack stamens in their flowers and can develop seedless fruit (Figure 6). Over-expression of VvHDZ27 in tomato also bears fruits with decreased numbers of seeds (unpublished data). These results demonstrate that HD-Zip family genes in grapes play pivotal roles in seed abortion. The endogenous SA content was increased in flowers at different stages, as was the relative expression of homologous genes of SARD and EDS in tomato (Figure 3). The results are consistent with the idea that VvHDZ28 positively regulates expression of the SA biosynthesis gene EDS1.

HD-Zip genes function as important growth regulators in plant development (Baima et al., 2001; Colette et al., 2015; Gao et al., 2016). In the present study, most members of HD-Zip transcription family had higher expression in TS, including VvHDZ28. Similar expression patterns were found in SA pathway genes. In addition, the SA response cis-elements were found in SA pathway genes. In contrast, high SA content induces the expression of VvHDZ28 in TS and VvHDZ28 was found to be a positive regulator of SA synthesis and to enhance the expression of downstream related PCD genes and to accelerate abortion of seed in TS by regulating VvEDS1 to control SA synthesis. VvHDZ28 can also increase embryo abortion in grapevine and transgenic tomato. This indicates VvHDZ28 plays important roles in seed development. Further investigations are necessary to establish how VvHDZ28 responds to cell death with SA signalling-induced embryo abortion.

**Experimental procedures**

**Plant materials and growth conditions**

Plants of Thompson Seedless (Vitis vinifera L.) and Pinot Noir (Vitis vinifera L.) were grown in the Germplasm unit of Northwest Agriculture and Forests University, and managed following standard local fertilization, irrigation and pest-management practices. In 2013 and 2014, berries of TS and PN were harvested, opened with a scalpel and the seeds removed using forceps. This was done at 20, 30, 40 and 50 days after full flowering (DAF) (full flowering is defined as when more than 75% caps are off). Seeds were immediately frozen in liquid nitrogen and stored at −80°C pending analysis. The fresh weights and longitudinal and transverse diameters of seeds and berries were measured using digital calipers. For each experiment, three biological replicates of 30 berries and seeds were used.

**Morphological analyses**

Developing seeds of TS and PN were harvested on 20, 30, 40 and 50 DAF. Tomato flower buds of OE-HDZ28 and WT were also taken at flower buds about 5 mm, green flowers about 9 mm and flowers at anthesis. Samples were embedded in paraffin (Chevalier et al., 2014) and sectioned at 8 to 10 µm thickness using a rotary microtome (RM2145; Leica, Wetzlar, Germany). Grape seed sections were stained in Ehrlich’s haematoxylin

**Figure 7** A proposed model of genetic and molecular interactions in the regulatory network during embryogenesis and embryo abortion in grape. In TS, VvHDZ28 up-regulate expression of VvEDS1 by binding to its promoter while up-regulate VvSARD1 in an indirect way; genes involved in SA biosynthesis were expressed at higher levels in TS. Consequently, higher levels of SA accumulated in TS, which we propose plays an important role in the PCD in seed abortion. In PN, SA has lower content possibly due to altered histone modifications (such as increased H3K27me3) in VvSARD1 and VvEDS1.
(Saichi, Shanghai, China); flower sections of tomato were stained with toluidine blue and mounted under a glass coverslip. Serial longitudinal and transverse sections were observed with a light microscope.

**Determination of concentrations of endogenous hormones**

Concentrations of endogenous hormones including ABA, IAA, JA, SA, aminocyclopropane-1-carboxylic acid (ACC, the precursor of ethylene) and cytokinins (tZ and tZR) in seeds of PN and TS were extracted as described by Müller and Munné-Bosch (Müller and Munné-Bosch, 2011). Simply, 50 mg seeds of each period were ground in powder into liquid nitrogen and then extracted with 800 μl of extraction solvent (for ABA, SA, JA and IAA methanol: isopropanol:glacial acetic acid, 20:79:1 (v/v/v), for cytokinins methanol:isopropanol:glacial acetic acid, 60:39:1 (v/v/v) and for ACC methanol:glacial acetic acid, 99:1 (v/v)). tZR (Z0375), tZ (20375), ACC (343412), ABA(A1046) and IAA(45553) were purchased from Sigma-Aldrich to prepare standard curves to quantify hormone concentrations. The hormone concentrations in the extracts were quantified using LC-20AT a high-performance liquid chromatography system (Shimadzu, Kinh Do, Japan) and an API 2000™ electrospray tandem-mass spectrometer (Allen-Bradley, Milwaukee, WI). Endogenous GA and BR (Brassinosteroids) in seeds were quantified using ELISA. Three biological replicates for each hormone were used.

**RNA extraction and qRT-PCR**

In 2013 and 2014, RNA was extracted from 200 mg seeds of PN and TS using the Plant RNA Kit (OMEGA). qRT-PCR assays were carried out to validate the RNA-Seq results with an independent technique. Total RNAs for qRT-PCR were from the same samples used for RNA-Seq. To this point, the expressions of 20 DEGs were analysed in PN and TS that were also sequenced by RNA-Seq (Table S1). cDNA synthesis and qRT-PCR were carried out as described previously (Xiang et al., 2013). SYBR Green was used to detect PCR products on a Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Gene expression was normalized using edgeR (Robinson et al., 2010). Gene expression was normalized using edgeR (Robinson et al., 2010). Expression values for the same germplasm at different times (false discovery rate [FDR] < 0.01, log2-fold change > 1). Principal component analysis was done using the pcomp command with the default parameters in the R software package.

**Chromatin immunoprecipitation (ChiP)**

ChiP was carried out as described previously (Kauffmann et al., 2010; Yang et al., 2015). Briefly, samples of about 1 g fresh weight of seeds were cross-linked with 1% formaldehyde under vacuum for 35 min and stored at −80°C. Later, seeds were ground to powder in liquid nitrogen; the chromatin complexes were isolated and sonicated, then incubated with 10 μg anti-acetyl-Histone H3 (Upstate; 06-599) antibody and anti-trimethyl-Histone H3 (Lys27, Millipore; 07-449) antibody. Equal amounts of sample without antibody were used as mock controls. The precipitated DNA was recovered and analysed by qPCR using specific primers listed in Table S1. Each ChiP value was normalized to its respective input DNA value. 100 μM exogenous SA was sprayed on berries of PN at DAF20, 30, 40 and 50. Seeds were detached post 1 h, 2 and 4 h. Then, RNA was extracted, and expression of HDZ28, SARD1 and EDS1 was detect by RT-PCR; three replicates were done for each gene.

**Dueal-Luciferase (Dual-LUC) assay**

2400 bp promoter sequence upstream of ATG for SARD1 and EDS1 were amplified and cloned into pGreen II 0800-LUC vector, generating pGreenII::VvSARD1 and pGreenII::VvEDS1. The full-length of VvHDZ28 was cloned and inserted into pGreenII-62SK, to generate pGreenII-62SK::VvHDZ28. Two constructs were transformed into Agrobacterium tumefaciens (strain GV3101). The assay was carried out using Hellens’ method (Hellens et al., 2005). Nicotiana benthamiana plants were grown in a glasshouse at 22°C and under natural light with daylight extension to 16 h. Dual-Luciferase Reporter Assay System (Beyotime) was used to detect LUC and REN fluorescent values following the assembly. Cuffmerge (version v2.2.1) (Trapnell et al., 2012) was used to merge the transcripts of PN and TS at four time points. The TS transcripts correspond to the PN transcripts using cuffcompare. Transcripts were filtered by (i) keeping Ensembl annotation gene; (ii) keep coding gene analyse by CPC (http://cpc.cbi.pku.edu.cn/, Coding Potential Calculator) (Kong et al., 2007); and (iii) keep CPM≥1. Proteins inferred from genes were classified based on their similarity (BLASTp, E-value ≤ 10−5) to sequences in the Swiss-Prot database. Gene boundaries were revised before gene estimate expression calculation as reads count by using bedtools (Quinlan and Hall, 2010). Gene expression was normalized using edgeR (Robinson et al., 2010). Gene expression was normalized using edgeR (Robinson et al., 2010).
manufacturers instructions. Three biological repeats were done for each experiment.

**GUS assay**

Promoters of VvSARD1 and VvEDSI were fused to pBI121 vector, and ORF of VvHDZ28 was fused to pCAMBIA2300-GFP binary vector under CaMV35 promoter. pSARD1::GUS, pEDSI::GUS and HDZ28::GFP were transformed into Agrobacterium tumefaciens (GV3101), and then, pEDSI::GUS and HDZ28::GFP or pSARD1::GUS and HDZ28::GFP were co-injected into the abaxial surfaces of tobacco leaves (Nicotiana benthamiana).

An 950 bp promoter sequence upstream of HDZ28 was cloned and inserted into pBI121 vector to generate HDZ28pro::GUS, and transformed into Agrobacterium tumefaciens (GV3101). The Agrobacterium cells containing pHDZ28 were then injected into the abaxial surfaces of tobacco leaves. GUS staining and quantitative assays of injected leaves were carried out following the procedure of Jefferson (Jefferson, 1987; Jefferson, 1988). All assays were repeated at least three times.

**Yeast one hybrid experiment**

pEDSI, pSARD1 and fragments for each promoter were inserted into pLacZ vector, resulting in the EDS1pro:LacZ and SARD1pro:LacZ reporter constructs. Full length of HDZ28 was cloned into the pJ4-5 vector to generate AD-HDZ28 (Lin et al., 2007). Yeast one-hybrid assay was performed according to the Yeast Protocols Handbook (Clontech).

**Production of VvHDZ28 transgenic tomato lines**

For VvHDZ28 over-expression, full-length cDNA of VvHDZ28 amplified from TS was inserted into a binary vector pBI121 vector under the transcriptional control of the cauliflower mosaic virus 35S promoter and the Nos terminator, to generate 35Spro::VvHDZ28::GUS. Transgenic tomato (Solanum lycopersicum cv. Micro-Tom) plants were generated by Agrobacterium tumefaciens-mediated transformation according to the procedure described above (Cortina and Culiánez-Maciá, 2004), and transformed lines (T0) were first selected on MS medium (kanamycin 80 mg L⁻¹) and then, rooted explants were planted in pots. Seeds of transgenic plants were harvested (T1) and sowed in MS medium containing 80 mg L⁻¹ kanamycin. Tomato was planted under standard greenhouse conditions. The conditions for the culture chamber were as follows: 14/10 h day/night cycle, 25/28°C day/night temperatures, 80% humidity and 250 mmol m⁻² s⁻¹ intensity luminosity. Agrobacterium tumefaciens-mediated transformation of VvHDZ28 into Micro-tom was repeated twice to avoid insertion-site influences.

**Molecular analysis of transformants**

Genomic DNA was isolated from 1 g young leaves of the putative transgenic tomato plantlets of kanamycin – resistant seedlings (T0). Specific primers in pBI121 vector (pBI121:F and pBI121:R, Table S1) were designed to identify VvHDZ28 in genomic DNA. GUS assay was carried out to detect expression of HDZ28 in different tissues. 10 μg DNA was digested with EcoR I and separated by electrophoresis on 1% agarose; southern blot analysis was carried out according previous reports.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

This project was designed by Y.X., Y.W. and Z.L. Transcriptome data were analysed by Z.L. and K.W. Transgenic plant phenotypes were analysed by X.Y., Z.L., C.Z., M.D and Y.X. Z.L. and X.Y. wrote and edited the paper. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Global Evaluation of the RNA-Seq Experiment. Figure S2 Selected example of gene boundary revised in RNA-Seq analysis. Figure S3 Gene start end extend length distribution. Figure S4 The scatter plot comparing the gene expression levels pairwise among the two replicates. Figure S5 GO enrichment and heat map genes involved in GO modules.
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Figure S7 Concentration of SA and expression of SA related genes in PN and TS.
Figure S8 Expression of HDZ28, SARD1 and EDS1 in response to SA treatment.
Figure S9 Dual-Luciferase and GUS assay of VvHDZ28 and pSARD1 or pEDS1.
Figure S10 Yeast one-hybrid assay testing the binding of HDZ28 to the SARD1 and EDS1 promoters.
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