

Genome-wide analysis of the cation/proton antiporter (*CPA*) super family genes in grapevine (*Vitis vinifera* L.)

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Abstract

Grapevine (*Vitis vinifera* L.) is sensitive to salinity. Cation/proton antiporter genes function in regulating ions and pH homeostasis in organisms, enhance salt resistance/tolerance of plants through the vacuolar compartmentalization of Na⁺, Na⁺ efflux from the cell, and affecting K⁺ concentrations. Two previous general bioinformatics studies on *CPA* gene families, including that of grapevine, showed different numbers of grapevine *CPA* genes because of using different genome assemblies. In this report, we employed comprehensive bioinformatics and annotation analysis and carefully re-evaluated the previous studies characterizing the *CPA* proteins. We resolved the discordance of *CPA* family genes in grapevine, and revealed that duplications contribute to expansion of *CPA* family genes in grapevine. Furthermore, we identified motifs between grapevine and *Arabidopsis* and found some motifs are subgroup-specific motifs. In addition, we investigated the gene structure among the *CPA1* subfamily genes in six species. In our analysis 29 *CPA* genes were identified in the grapevine reference genome. This detailed information on the *CPA* superfamily in the physiological responses to salinity and osmotic stress and for potential development of salt resistant cultivars.

Keywords: *CPA* gene super-family, grapevine, cation/proton antiporter 1, NhaP, NHX.

Abbreviations: CPA, Cation/proton antiporter; NHX, Na⁺/H⁺ exchanger; Nhap, Na⁺/H⁺ antiporter; KEA, K⁺-efflux antiporter; CHX, cation/H⁺ exchanger;

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important economic crops worldwide. In 2010 it is grown on approximately 7.1 million hectares (Bouby et al., 2013). The cultivated grapevine cultivars have been shown to adapt to semiarid environments and are considered moderately tolerant to salinity (Gil et al., 2013; Hawker and Walker, 1978; Shani et al., 1993; Walker et al., 2002). Because of climate changes and mismanagement of irrigation with ground water, salinity is becoming an increasingly significant issue in global viticulture (Cramer et al., 2011). The studies of grapevine salt tolerance have traditionally focused on selections of salt-tolerant rootstocks, physiological comparison of salt tolerance in different grapevine cultivars (Antcliff et al., 1983; Oki and Lieth, 2004) and development of a high throughput assay (Hopper et al., 2014), but no have characterized the cation/proton antiporter (*CPA*) super-family and their roles in salt tolerance in grapevine. The *CPA* proteins primarily transport monovalent cations across membranes in maintaining a low Na⁺ concentration in the cytoplasm by reducing Na⁺ influx, Na⁺ efflux, and Na⁺ compartmentation (Bassil and Blumwald, 2014; Niu et al., 1995; Tester and Davenport, 2003), therefore, *CPA* functions primarily as couplers of the efflux of diverse monovalent cations with movement of protons (Brett et al., 2005; Davies, 1986; Fujisawa et al., 2007).

The *CPA* protein family has been divided into two major subfamilies, *CPA1* (2.A.36) and *CPA2* (2.A.37) (<http://plantst.genomics.purdue.edu/classification.shtml>) (Saier, 2000) based on their phylogenetic relationships (Brett et al., 2005; Chanroj et al., 2012; Maser et al., 2001; Ye et al., 2013). The *CPA1* can be further classified into the *NhaP* and *NHX* subfamilies, and is involved in salt exclusion at the plasma membrane of root cells and/or salt compartmentalization at the tonoplast of the leaf cell vacuoles (Apse et al., 1999; Shi et al., 2003; Sze et al., 1999), therefore, effectively preventing accumulation of potentially toxic Na⁺ into the endosomal lumen (Blumwald and Poole, 1985; Gorham et al., 1985; Greenway and Munns, 1980; Zhang et al., 2012b). Based on the previous studies, the *NhaP* subfamily was often classified into the *NHX* gene family due to its similarity to the *NHX* family and a limited number of genes (Chanroj et al., 2012; Gorham et al., 1985; Maser et al., 2001). However, significant differences have been found between the *NhaP* and *NHX* subfamilies (Chanroj et al., 2012; Rodriguez-Rosales et al., 2009). *NhaPs* are located in the plasma membrane and the protein sequences are remarkably long, with more than 600 residues, have a particularly long C-terminal tail that specifically recognizes Na⁺; a typical member is *Arabidopsis SOS1* (An et al., 2007; Katiyar-Agarwal et al., 2006; Oh et al., 2010; Qi and Spalding, 2004; Qiu et al., 2003; Qiu et al., 2002; Quintero et al., 2011; Shi et

al., 2002). However, there is controversy about the *Arabidopsis SOS1*, some studies suggested *AtSOS1* belonged to the NHX-type transporters, and was named *AtNHX7* (Fu et al., 2012; Zhang et al., 2012b). The *NHX* family; however, is a Na^+/H^+ exchanger family that can be divided into two categories, the PM (plasma membrane) group and the IC (intracellular) group (Rodriguez-Rosales et al., 2009). The first *NHX* gene was discovered in *Arabidopsis* and named *AtNHX1*, which plays an important role in tolerance to salt and drought (Gaxiola et al., 1999). Since then, more *NHX* members have been identified in *Oryza sativa* (Fukuda et al., 2004), *Populus euphratica* (Ye et al., 2009), *Solanum lycopersicum* (Galvez et al., 2012), *Zea mays* (Zorb et al., 2005), *Glycine max* (Chen et al., 2014), *Dendranthema morifolium* (Zhang et al., 2012a) and *Ipomoea nil* (Ohnishi et al., 2005). Most *NHX* family proteins have 10-12 transmembrane structures, about 550 amino acid residues, and a putative amiloride-binding domain (FF(IL)(Y/F) LFLPP) in the third transmembrane region (Darley et al., 2000; Hanana et al., 2007; Putney et al., 2002; Reguera et al., 2014; Yamaguchi et al., 2003). But, not all of the members have these characteristics, for example, *AtNHX5* only has 9 transmembrane structures and in maize, *GRMZM2G013627_P02* only has 383 AA residues (Chanroj et al., 2012; Reguera et al., 2014). Plant NHX-type genes have been showed to be involved in many cellular process, including transport of the K^+ and Na^+ ions into vacuoles (Pardo et al., 2006, Zhang and Blumwald, 2001), and maintain the pH of during the fruit development of grapevine (Hanana et al., 2007). To thoroughly understand how the *CPA* genes play roles in physiological process and salt tolerance in grapevine, one fundamental issue we need to resolve is how many *CPA* genes are there in the grapevine reference genome. Chanroj et al. (2012) included 27 grapevine *CPA* genes in their overall *CPA* evolutionary study, while Ye et al. (2013) enlisted 31 grapevine *CPA* genes in their networking study. This difference is at least partially caused by using the different standards in naming and classifying the *CPA* genes. In this paper, we have employed several bioinformatics analysis tools and carefully re-evaluated the previous studies in characterizing the *CPA* proteins and gene annotation methods, and concluded that grapevine *CPA1* gene family contained 29 *CPA* genes, therefore, resolving the disagreement in earlier studies. This detailed information on the *CPA* superfamily in grapevine lays the foundation for further characterization of these grapevine *CPA* genes for their roles in the physiological processes.

Results and Discussion

Resolving the discordance of *CPA* super family genes in grapevine

Grapevine genome contains 29 *CPA* genes (Table 1), which is different from previous studies (Chanroj et al., 2012; Ye et al., 2013). The detail of the difference between the studies and our result are showed in Table 2. We identified 4 more *CPA* genes than Salil's study (Chanroj et al., 2012). The additional genes (*VIT_02s0025g00800.t01*, *VIT_15s0024g00280.t01*, *VIT_15s0024g00260.t01* and *VIT_02s0025g00790.t01*) were confirmed to be located on their respective chromosomes and contained the PF00999 domain. We excluded two genes (*GSVIVT01024625001*, *GSVIVT01030261001*) from another study (Ye et al., 2013) because these two genes contained no PF00999 domain. To make sure the accuracy of our results we did a search of paralogs for each

group of transporters using the Gramene lists for the *Vitis* V2 annotation from Gramene (<http://www.gramene.org>), the result showed one more gene than our first result. It is *VIT_15s0046g03380*. However, when we checked it from PFAM, we found it did not contain any domain, so it was not included in our result. The locations of *VviCPA* genes were given a representation based on the grapevine genome annotation (12× V1 assembly), which was verified with RNA-seq data, at CRIBI (Fig. 1). Twenty-six out of 29 *VviCPA* genes were mapped to 14 out of 19 chromosomes (Chr). The distribution of *VviCPA* genes was uneven across all of the chromosomes. Five (19.23%) *VviCPA* genes were located in Chr 2; four (15.38%) *VviCPA* genes were located in Chr 6; three *VviCPA* genes were located in Chr 15; Chr 5, 8 and 14 had two *VviCPA* genes, respectively; Chr 1, 4, 7, 10, 11, 13, 16 and 19 each had one *VviCPA* gene. But no genes were located in Chr 3, 9, 12, 17 and 18. More genes (18, 69.23%) were located in the end positions of chromosomes than in the middle. As previously reported (Rockman et al., 2010), this can be inferred that the *VviCPA* family might have experienced more variations during the grapevine evolution. Moreover, we further identified the duplication events based on the chromosome locations of 29 grapevine *CPA* genes. Genes which have physical locations within a 100-kb adjacent region in individual chromosomes were identified as tandem duplication, mainly contributing to the expansion of *CPA2* subfamily, with 11 genes in four tandem clusters within a 100-kb genomics region on chromosome 2, 6, 8 and 15, respectively (Fig. 1). However, the previous study (Ye et al., 2013) had the 12 *CPA* genes in tandem duplication blocks, including the *GSVIVT01024625001*. In an effort to gain further insight into the evolutionary history of grapevine *CPA* genes, we analyzed the comparative synteny map between grapevine and *Arabidopsis* genomes. Because the functions of most *Arabidopsis* *CPA* genes have been well studied, we may infer the functions of grape *CPAs* based on their *Arabidopsis* orthologues. Nineteen *CPA* orthologous pairs were identified between grapevine and *Arabidopsis* genomes (Fig. 2, Table 3), suggests that they might have already existed before the split of grapevine and *Arabidopsis*. The existence of one triplet (*VIT_14s0030g00710/VIT_07s0104g01280/VIT_05s0020g01960*) in a syntenic block supports the fusion hypothesis of the grapevine genome (Jaillon et al., 2007; Malacarne et al., 2012).

Classifications and Characteristics of *VviCPA1* family

We constructed the phylogenetic tree by including five additional species in the tree of plant life to obtain better perspective of the grapevine *CPA* gene classification. This phylogenetic tree was built on the 173 non-redundant genes encoding putative *CPA* proteins from six species (Fig. 3, Table 4). All *CPA* genes could be divided into five groups, group-I to V. The detailed characteristics of 29 members of the grape *CPA* were also showed in Table 1. The gene structures, conserved domains and transmembrane structures are shown in Fig 4. Notably, three (*VIT_02s0025g0800.t01*, *VIT_15s0024g00260.t01* and *VIT_02s0025g00790.t01*) had less than 127 AA in the conserved domain, indicating that the Na^+/H^+ exchanger domain of the three genes was less than 1/3 HMM. The *VIT_00s0282g00020.t01* did not contain any transmembrane structure, although the conserved domain (Na^+/H^+ exchanger domain) had 384 amino acids residues. So it might be inferred that these genes could not perform the complete functionality of Na^+/H^+ exchanger domain, consequently, the function of transmembrane protein.

Table 1. CPA genes in grapevine.

Gene ID	Chr	Locus		Protein length	HMM length	Number of TM	Number of exon
		Start	End				
VIT_16s0022g02060	chr16	14445300	14477431	577	369	11	20
VIT_11s0016g02400	chr11	1922308	1942840	522	369	10	20
VIT_02s0025g00780	chr2	798760	802286	796	184	12	3
VIT_05s0020g01150	chr5	2902893	2906110	802	387	10	3
VIT_02s0025g00820	chr2	814778	818141	787	385	10	3
VIT_04s0044g01470	chr4	22994818	22997726	837	384	12	3
VIT_08s0007g00030	chr8	14416489	14420425	844	396	11	5
VIT_02s0025g00810	chr2	809315	812187	786	384	12	4
VIT_00s0282g00020	chrUn	21024160	21038613	563	384	0	14
VIT_08s0007g00020	chr8	14398881	14405247	826	389	10	4
VIT_15s0046g03390	chr15	19994041	20009585	612	370	11	19
VIT_01s0011g06550	chr1	6328905	6391280	1141	413	12	23
VIT_14s0128g00020	chr14	2600669	2606953	541	412	10	14
VIT_06s0004g07480	chr6	8277395	8280258	784	380	10	3
VIT_05s0020g01960	chr5	3677507	3683743	541	411	10	15
VIT_19s0090g01480	chr19	7519251	7525059	521	415	11	14
VIT_14s0030g00710	chr14	4886251	4918304	539	415	9	13
VIT_10s0003g03030	chr10	5163355	5167643	913	378	12	4
VIT_06s0004g07400	chr6	8163557	8166092	783	384	10	3
VIT_06s0009g00990	chr6	12257673	12260421	781	386	10	4
VIT_00s0577g00030	chrUn	32187188	32190763	767	387	9	5
VIT_06s0004g07470	chr6	8264471	8267029	780	357	12	2
VIT_15s0024g00280	chr15	371594	385067	315	184	6	13
VIT_07s0104g01280	chr7	2309198	2315931	499	211	11	14
VIT_02s0025g00800	chr2	807303	808096	193	109	4	2
VIT_13s0064g00620	chr13	22356493	22359290	714	333	9	4
VIT_00s0577g00040	chrUn	32193775	32195904	537	163	5	3
VIT_15s0024g00260	chr15	357749	363233	242	75	5	9
VIT_02s0025g00790	chr2	805563	806989	196	97	3	3

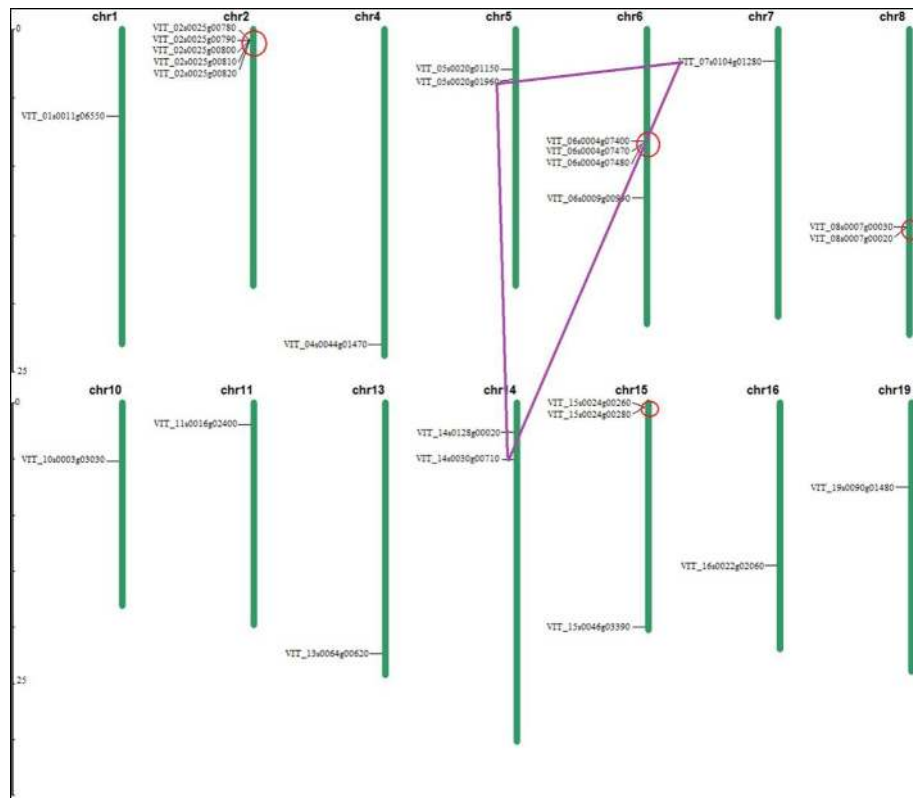


Fig 1. The chromosome locations of 26 *VvCPAs* on 14 chromosomes. The red cycle means tandem duplications, and the violet circle means triplet in a syntenic block.

Table 2. The comparison of *VviCPA* genes between previous studies and this study.

This paper		Conserved and diversified gene families of monovalent cation/H+ antiporters from algae to flowering plants		Comparative analysis of cation /proton antiporter superfamily in plants	
Database: CRIBI		Database: Phytozome			
Gene ID	Locus	Gene ID	Locus	Gene ID	Locus
VIT_16s0022g02060	chr16:14,444,948..14,477,536	GSVIVT0101 8483001	chr16:14,444,939..14,477,574	GSVIVT0101 8483001	chr16:14,444,939..14,477,574
VIT_11s0016g02400	chr11:1,922,155..1,943,295	GSVIVT0101 5222001	chr11:1,922,070..1,943,368	GSVIVT0101 5222001	chr11:1,922,070..1,943,368
VIT_02s0025g00800	chr2:806,990..808,096	-----	-----	GSVIVT0101 9457001	chr2:807,009..808,096
VIT_00s0282g00020	chrUn:21,024,160..21,038,616	GSVIVT0100 5667001	chrUn:21,024,004..21,038,616	GSVIVT0100 5667001	chrUn:21,024,004..21,038,616
VIT_15s0046g03390	chr15:19,993,956..20,009,801	GSVIVT0102 6846001	chr15:19,993,952..20,009,808	GSVIVT0102 6846001	chr15:19,993,952..20,009,808
VIT_15s0024g00280	chr15:371594..385070	-----	-----	GSVIVT0101 9361001	chr15:370,963..405,661
VIT_07s0104g01280	chr7:2309018..2315931	GSVIVT0101 1001001	chr7:2,308,849..2,316,652	GSVIVT0101 1001001	chr7:2,308,849..2,316,652
VIT_05s0020g01960	chr5:3677002..3684070	GSVIVT0101 7814001	chr5:3,676,950..3,684,048	GSVIVT0101 7814001	chr5:3,676,950..3,684,048
VIT_14s0030g00710	chr14:4886251..4918649	GSVIVT0102 1972001	chr14:4,884,368..4,918,654	GSVIVT0102 1972001	chr14:4,884,368..4,918,654
VIT_14s0128g00020	chr14:2600592..2606971	GSVIVT0100 0002001	chr14:2,600,404..2,607,034	GSVIVT0100 0002001	chr14:2,600,404..2,607,034
VIT_19s0090g01480	chr19:7518928..7525059	GSVIVT0103 7753001	chr19:7,518,931..7,525,059	GSVIVT0103 7753001	chr19:7,518,931..7,525,059
VIT_01s0011g06550	chr1:6328691-6391634	GSVIVT0101 1573001	chr1:6,328,679..6,391,646	GSVIVT0101 1573001	chr1:6,328,679..6,391,646
VIT_15s0024g00260	chr15:357595..363236	-----	-----	GSVIVT0101 9363001	chr15:357,536..363,268
VIT_00s0577g00040	chrUn:32,193,772..32,195,904	GSVIVT0100 7481001	chrUn:32,187,185..32,190,760	GSVIVT0100 7481001	chrUn:32,187,185..32,190,760
VIT_00s0577g00030	chrUn:32,187,185..32,190,763	GSVIVT0100 7482001	chrUn:32,193,772..32,195,904	GSVIVT0100 7482001	chrUn:32,193,772..32,195,904
VIT_13s0064g00620	chr13:22,356,493..22,359,293	GSVIVT0103 2132001	chr13:22,355,860..22,359,293	GSVIVT0103 2132001	chr13:22,355,860..22,359,293
VIT_06s0009g00990	chr6:12,257,670..12,260,421	GSVIVT0103 7524001	chr6:12,257,670..12,260,421	GSVIVT0103 7524001	chr6:12,257,670..12,260,421
VIT_02s0025g00780	chr2:798,176..802,286	GSVIVT0101 9454001	chr2:798,757..804,686	GSVIVT0101 9454001	chr2:798,757..804,686
VIT_02s0025g00820	chr2:814,661..818,141	GSVIVT0101 9459001	chr2:814,055..819,061	GSVIVT0101 9459001	chr2:814,055..819,061
VIT_02s0025g00810	chr2:809,083..813,431	GSVIVT0101 9458001	chr2:809,083..813,431	GSVIVT0101 9458001	chr2:809,083..813,431
VIT_02s0025g00790	chr2:805,237..806,989	-----	-----	GSVIVT0101 9456001	chr2:805,239..806,989
VIT_05s0020g01150	chr5:2,902,757..2,906,283	GSVIVT0101 7721001	chr5:2,902,757..2,906,309	GSVIVT0101 7721001	chr5:2,902,757..2,906,309
VIT_08s0007g00030	chr8:14,416,470..14,420,425	GSVIVT0103 4209001	chr8:14,416,399..14,420,572	GSVIVT0103 4209001	chr8:14,416,399..14,420,572
VIT_08s0007g00020	chr8:14,398,754..14,405,247	GSVIVT0103 4211001	chr8:14,398,774..14,405,247	GSVIVT0103 4211001	chr8:14,398,774..14,405,247
VIT_04s0044g01470	chr4:22,994,815..22,997,726	GSVIVT0102 6473001	chr4:22,994,815..22,997,726	GSVIVT0102 6473001	chr4:22,994,815..22,997,726
-----	-----	-----	-----	GSVIVT0103 0261001	chr8:9,772,720..9,780,613
-----	-----	-----	-----	GSVIVT0102 4625001	chr6:8,281,283..8,286,829

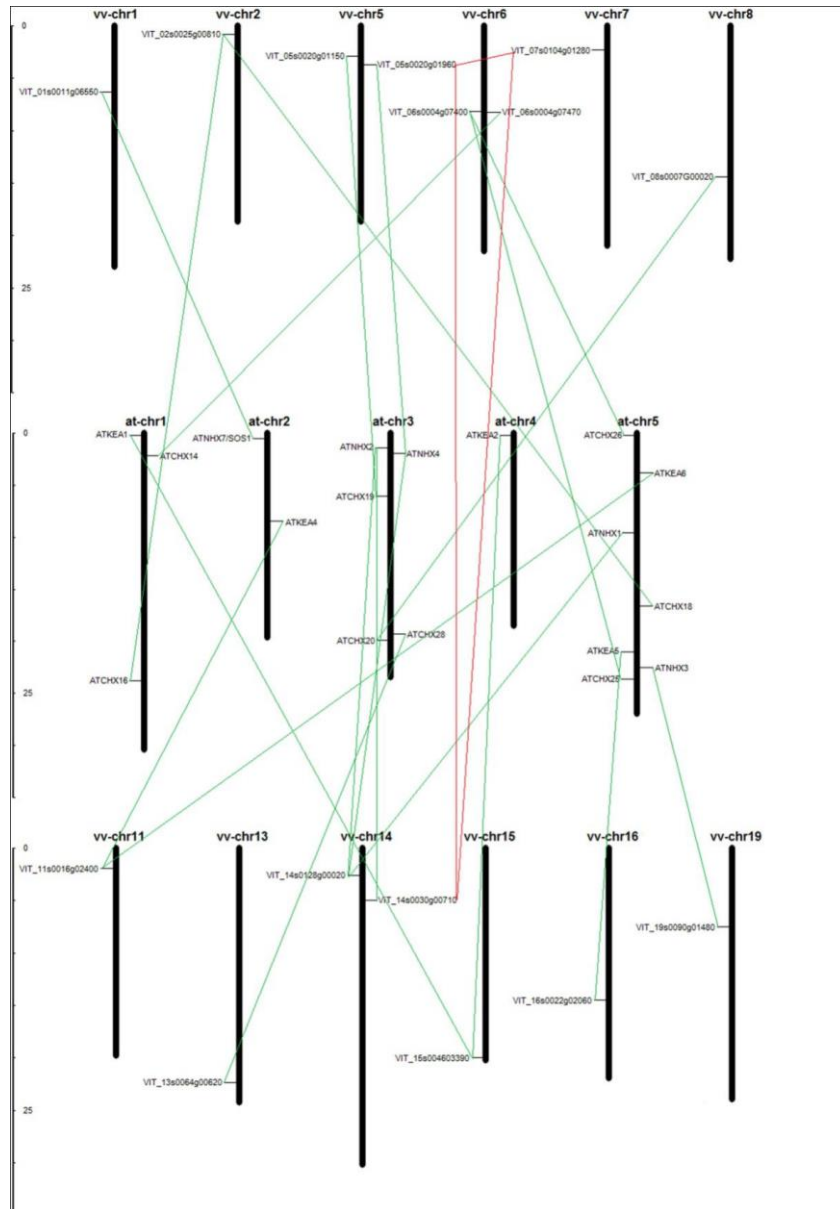


Fig 2. Synteny related to CPAs in grapevine and Arabidopsis. The green lines mean orthologous pairs.

Whether these genes have full or partial functions requires additional functional characterizations.

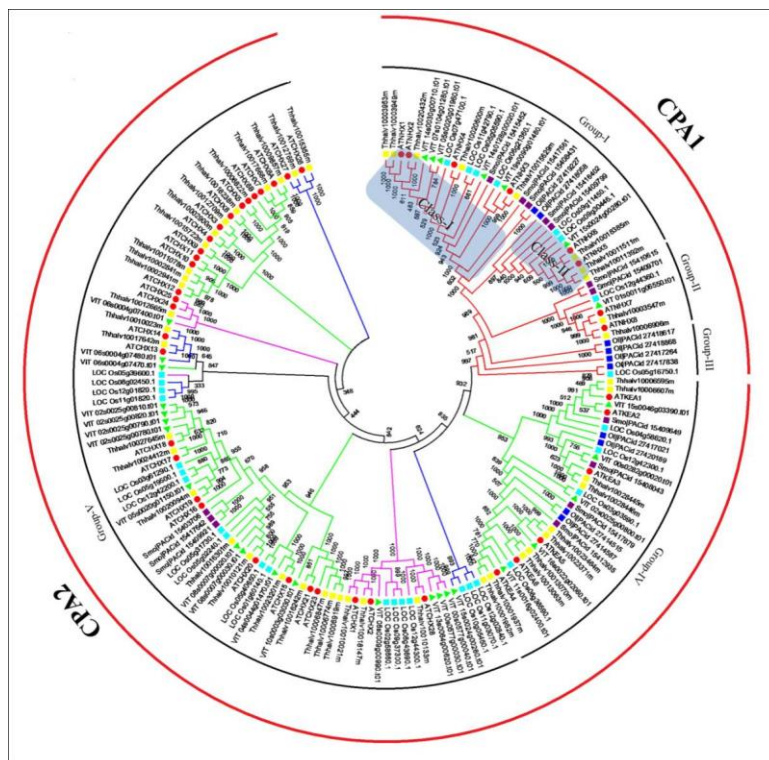
The Group-I was the *CPA1* gene family and they were used to make a comparative study on the structure (Fig. 5). Since the *CPA1* genes of the *CPA* superfamily have been widely studied in *Arabidopsis* and other species, we have further analyzed the *CPA1* genes in grapevine, which consists of seven genes (Fig. 3). Among the seven *VviCPA1* genes, *VIT_01s0011g06550* was a member of *NhaP* subfamily, and *VIT_15s0024g00280*, *VIT_07s0104g01280*, *VIT_05s0020g01960*, *VIT_14s0128g00020*, *VIT_14s0030g00710* and *VIT_19s0090g01480* belong to the *NHX* subfamily.

The Group-I can also be further divided into class-I and class-II (Bassil et al., 2011; Brett et al., 2005; Yokoi et al., 2002). All the 5 members of class-I (*VIT_07s0104g01280*, *VIT_05s0020g01960*, *VIT_14s0128g00020*, *VIT_14s0030g00710*, and *VIT_19s0090g01480*), contained an amiloride-binding domain (FFI/LY/FLLPPI), and the position was conserved, at the 3rd TM domain. There is one class-II gene

in grapevine (*VIT_15s0024g00280*), which didn't contain a putative amiloride-binding domain (Table 5). Previous studies suggested that all NHE-like Na^+/H^+ transporters have an amiloride-binding domain (Harris and Fliegel, 1999; Yun et al., 1993), but our study based on all *CPA1* genes of the six species showed that the position of this domain was not conserved, and many *NHXs* genes don't contain the amiloride-binding domain, including the *VIT_15s0024g00280* (Table 5). Many studies focusing on the function of *AtNHXs* suggested that the class-1 and class-2 showed different locations, structures and different functions (Aharon et al., 2003; Yamaguchi et al., 2003). *VIT_05s0020g01960*, *VIT_19s0090g01480* and *VIT_14s0128g00020* were predicted to be localized in the vacuole membrane (Hanana et al., 2007) (<http://genomes.cribi.unipd.it/grape/>). This prediction may be accurate because *Arabidopsis* paralogs genes such as *AtNHX1-4* (Apse et al., 1999; Wang et al., 2007; Yokoi et al., 2002) are also localized in the vacuole

Table 3. Synteny related to *CPA* genes in grapevine and Arabidopsis.

Duplicated gene	Duplicated gene	subfamily	Ka	Ks
VIT_01s0011g06550	ATNHX7	NahP	0.24	1.25
VIT_14s0128g00020	ATNHX2	NHX	0.30	3.80
VIT_14s0128g00020	ATNHX4	NHX	0.35	-1.00
VIT_14s0128g00020	ATNHX1	NHX	0.32	-1.00
VIT_19s0090g01480	ATNHX3	NHX	0.19	1.79
VIT_05s0020g01960	ATNHX4	NHX	0.20	2.33
VIT_14s0030g00710	ATNHX2	NHX	0.15	1.89
VIT_11s0016g02400	ATKEA4	KEA	0.08	1.35
VIT_11s0016g02400	ATKEA6	KEA	0.11	1.77
VIT_15s0046g03390	ATKEA1	KEA	0.06	1.06
VIT_15s0046g03390	ATKEA2	KEA	0.05	1.03
VIT_16s0022g02060	ATKEA5	KEA	0.12	1.17
VIT_13s0064g00620	ATCHX28	CHX	0.45	2.86
VIT_02s0025g00810	ATCHX16	CHX	0.41	-1.00
VIT_02s0025g00810	ATCHX18	CHX	0.32	-1.00
VIT_06s0004g07400	ATCHX25	CHX	0.50	-1.00
VIT_06s0004g07400	ATCHX26	CHX	0.88	-1.00
VIT_06s0004g07470	ATCHX14	CHX	0.69	-1.00
VIT_08s0007g00020	ATCHX20	CHX	0.27	-1.00
VIT_05s0020g01150	ATCHX19	CHX	0.20	-1.00

**Fig 3.** The phylogenetic tree of *CPAs* in the genomes of six species.

membrane; this suggests that the *VIT_05s0020g01960*, *VIT_19s0090g01480* and *VIT_14s0128g00020* should be able to execute the function same as *AtNHX1-4*. Group-II genes were orthologous with *Arabidopsis AtNHX7 (AtSOS1)*, in which no algae gene was found, suggesting that members of this group might have evolved with the emergence of terrestrial plants. They have more exons (19-23) than other members of the *CPA I* subfamily. Furthermore, the gene and protein structures were relatively conserved, except *AtNHX8*. Each member contained at least two low complexity regions, one of which was in the C-terminal. However,

Thhalv10006906m and *AtNHX8* contained a cNMP domain in the left of the Na^+/H^+ exchanger domain (Fig. 5). Previous studies suggested although group-I and group-II belonged to one gene family, they have different paths in the molecular evolution in the stress tolerance process (Pardo et al., 2006; Pires et al., 2013; Shi et al., 2000). We found the two groups had different evolutionary rate, which is corresponding with the previous results (Table 3). The average K_a/K_s between group-I (~0.0875) and group-II genes (~0.192), suggested that the older genes (group-I) with larger K_s value, had a slower evolution process (Pardo et al., 2006).

Table 4. The CPA in genomes of six species.

Species	Total genes	Size of	Number of CPA	Proportion of CPA
<i>Arabidopsis thaliana</i>	33602	135	42	0.125
<i>Theilingiella halophila</i>	28457	243.1	48	0.168
<i>Vitis vinifera</i>	26346	487	29	0.110
<i>Oryza sativa</i>	49061	372	30	0.061
<i>Selaginella</i>	22285	212.5	14	0.063
<i>Ostreococcus</i>	7791	13.2	10	0.128

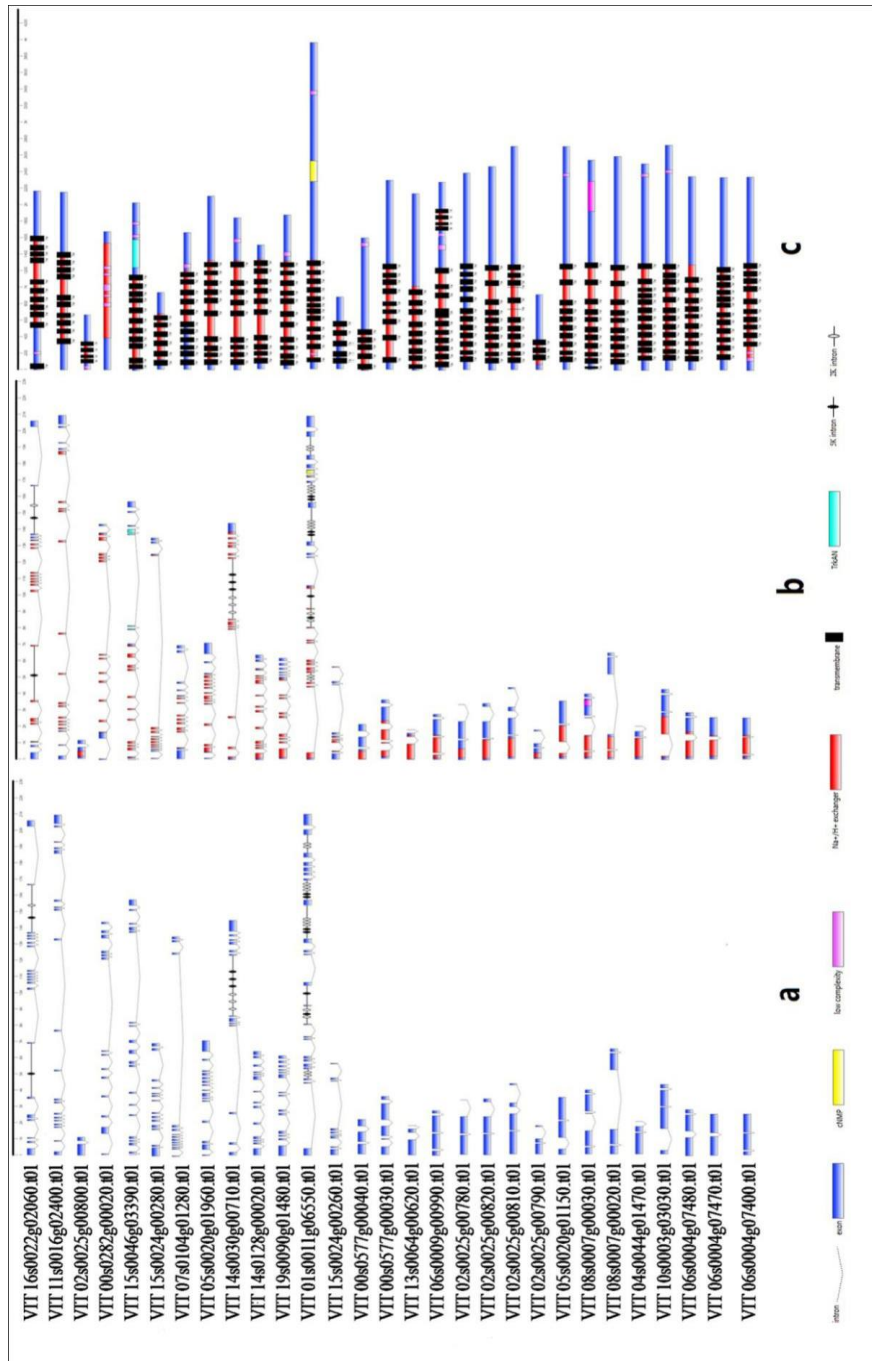


Fig 4. Exon-intron structure, conserved domains and transmembrane structure of *V. vinifera* CPAs. (a) Exons and introns are indicated by blue block and thin line, respectively. (b) Domains and exons are indicated by boxes. Different domains are indicated by different color denoted at the right bottom corner. (c) Transmembrane structures of 29 CPA proteins are indicated by black blocks.

Table 5. Amiloride binding domains in *NHX* genes.

Gene	Amiloride binding site	Location
Thhalv10003953m	88FFIYLLPPI96	3th TM
Thhalv10003949m	88FFIYLLPPI96	3th TM
ATNHX1	82FFIYLLPPI90	3th TM
ATNHX2	84FFIYLLPPI92	3th TM
Thhalv10020432m	88FFIYLLPPI96	3th TM
VIT_07s0104g01280.t01	82FFIYLLPPI90	3th TM
VIT_05s0020g01960.t01	85FFIYLLPPI93	3th TM
LOC_Os07g47100.1	85FFIYLLPPI93	3th TM
ATNHX4	86FFIYLLPPI94	3th TM
Thhalv10020600m	86FFIYLLPPI94	3th TM
LOC_Os11g42790.1	85FFIYLLPPI93	3th TM
LOC_Os05g05590.1	85FFIYLLPPI93	3th TM
VIT_14s0128g00020.t01	87FFIYLLPPI95	3th TM
Smo PACid:15415452	88FFIYLLPPI96	3th TM
LOC_Os06g21360.1	87FFIYLLPPI95	3th TM
VIT_19s0090g01480.t01	79FFIYLLPPI87	3th TM
ATNHX3	82FFIYLLPPI90	3th TM
Thhalv10015629m	82FFIYLLPPI90	3th TM
Smo PACid:15417561	NO	---
Smo PACid:15408431	NO	---
Ol PACid:27419227	NO	---
Ol PACid:27418058	NO	---
Smo PACid:15416402	NO	---
Smo PACid:15409799	87FFLFLPPI96	3th TM
LOC_Os09g11450.1	NO	---
LOC_Os09g30446.1	NO	---
VIT_15s0024g00280.t01	NO	---
ATNHX6	89 FFLFLPPI98	3th TM
Thhalv10018385m	88 FFLFLPPI97	3th TM
ATNHX5	88 FFLFLPPI97	3th TM
Thhalv10011511m	NO	---
Thhalv10011392m	NO	---

Group-III included five genes with relatively distant evolutionary relationships, including four green algae genes and one rice gene. The *LOC_Os05g16750.1* contained only two exons, which was a special case in terrestrial plants, and which conserved domain only contained 44 amino acids and a transmembrane domain. So it is likely that these are nonfunctional pseudo genes. No *CPA* gene in grapevine belongs to this group. The members of group-IV and group-V all belonged to the *CPA2* subfamily. Based on the study of *Arabidopsis*, the *CPA2* was divided into two categories, the K⁺-efflux antiporter (*KEA*) and cation/H⁺ exchanger (*CHX*) families. In this study, the group-IV is *KEA*, which included 5 grapevine genes (Fig. 3), and the group-V contained 17 grapevine genes (Fig. 3). The number of *CPA* gene increased with evolution from the lower plants to higher plants, which indicates that there was *CPA* gene expansion in the higher plant genomes. The two lower plants, *O. lucimarinus* and *S. moellendorffii*, had less *CPA* genes than other higher plant species. It is interesting that the *CPA* gene numbers of these two lower species are similar; the genome size of *S. moellendorffii* is over 16-fold of that of *O. Lucimarinus*, *V. vinifera* and *O. sativa* had similar gene numbers and genome sizes. In addition, *T. halophila*, a halophyte, had the largest number of *CPA* genes (48 *CPA* genes), whereas, *A. thaliana* had similar genes (42 *CPA* genes), which its genome was about half that of *T. halophila*'s. Furthermore, the percentages of *CPA* genes in the genomes (Table 4) indicated little relationship between the number of *CPA* genes and the

genome sizes, as seen in many other gene families (Chanroj et al., 2012; Lijavetzky et al., 2003). This may suggest that these plants in different deep lineages use mechanisms other than the gene dosage of these genes for regulating ion concentrations. In addition, we investigate the motifs with *e* value $1e-10$ (Ye et al., 2013) in *CPA1* genes of grapevine and *Arabidopsis* (there are 15 genes). The results showed (Table S1) that motif 8 was found in all *CPA1* genes of grape and *Arabidopsis*. The location of motif 8 is group-specific, it was located at the 3th TM from C-term in group-I, and near the N-term in group-II. In addition, some of the motifs were found to be group-specific, for example, two motifs were only found in genes of class-I, two motifs only in class-II and six motifs in Group-II, demonstrating the structure of proteins were very similar among the same group. The result might be suggested the evolution within group is very conservative.

Promoter analysis of the grapevine *CPA1* subfamily

The 1000-bp promoter regions of *VviCPA1* genes at the 5-end of the cDNA was analyzed by using the PLACE promoter analysis program (Higo et al., 1999) (www.dna.affrc.go.jp/PLACE/). The elements that were presented in each *VviCPA1* gene were shown in Table 6. Among the 12 elements, two were involved in abiotic stress MYCCONSUSAT was a recognition site of MYC, induced by cold and drought, and GT1GMSCAM4 was a

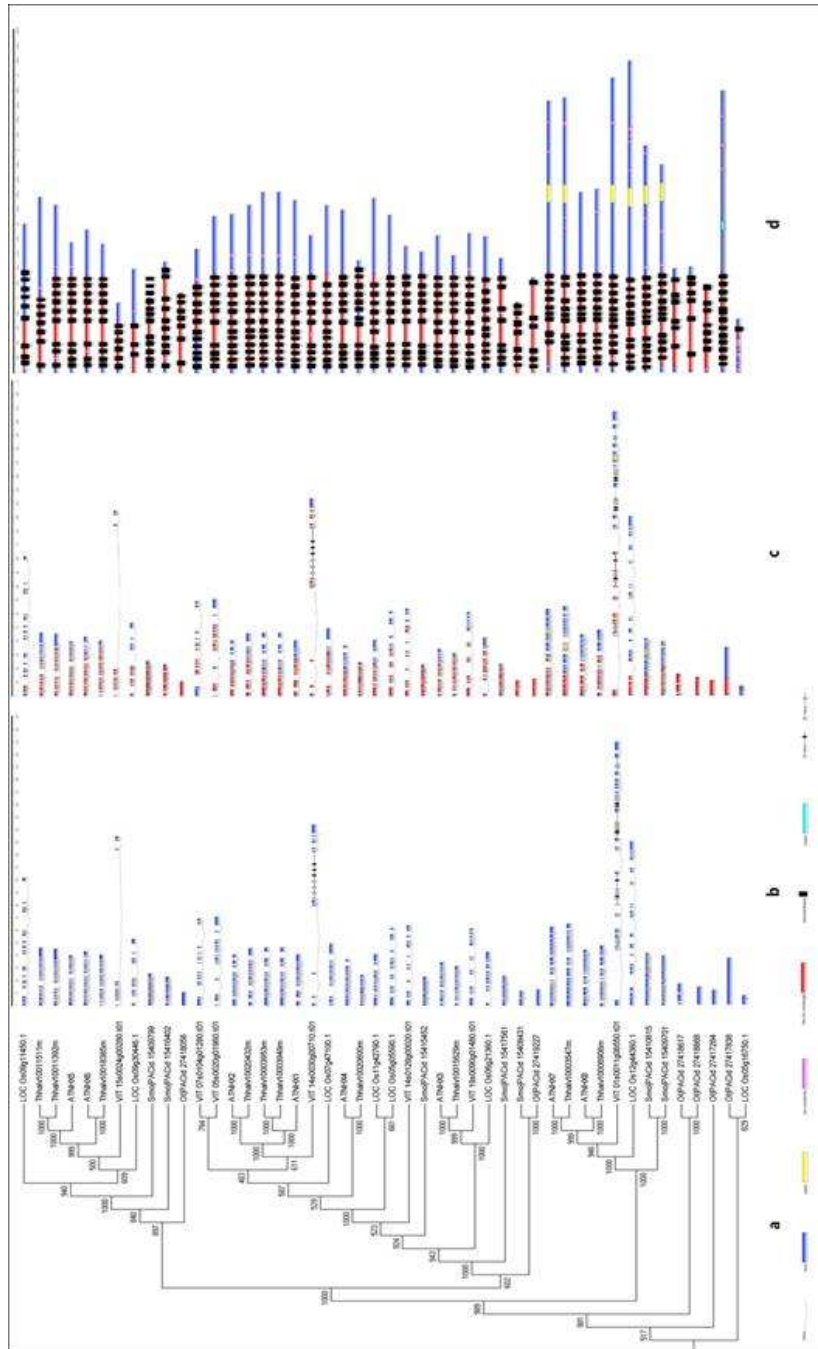


Fig 5. Phylogenetic relationship, conserved domains and transmembrane structure of *CPA1s* in six species. (a) The tree was part of the Phylogenetic tree of *CPAs*, which include 46 *CPA1* members. (b) Exons and introns are indicated by blue block and thin line, respectively. (c) Domains and exons are indicated by boxes. Different domains are indicated by different color denoted at the right bottom corner. (d) Transmembrane structures of 46 *CPA1* proteins are indicated by black block.

salt-responsive element, involved in pathogen- and salt-induction (Park et al., 2004). The existence of the two elements can be used as evidence that *VviCPA1s* may respond to salt stress.

Materials and Methods

Data acquisition and nomenclature

The CPA protein sequences of different organisms (*Ostreococcus lucimarinus*, *Thellungiella halophila*, *Selaginella moellendorffii*, *Oryza sativa*) were downloaded

from Phytozome v9.1 (www.Phytozome.net), CPA protein sequences of *Arabidopsis thaliana* were downloaded from the TAIR10 Genome Release (www.arabidopsis.org), and the *V. vinifera* sequences were from Proteome (<http://genomes.cribi.unipd.it/>) (12X genome coverage, release V1). The Hidden Markov Model (HMM) profile of CPA Na⁺/H⁺ exchanger domain (PF00999) was downloaded from Pfam (<http://pfam.sanger.ac.uk/>) and was then employed as a query to search against all proteins using the program HMMER3.0 (Eddy, 1998) with confidence (E-value < 1.0). All output genes were manually checked, and the predicted genes, which did not have the Na⁺/H⁺ exchanger domain, were rejected.

Table 6. The main cis-element analysis of *VvCPA1* genes promoter sequences.

Element name	Numbers of each member contains						
	VIT_14s01 28g00020	VIT_07s01 04g01280	VIT_05s00 20g01960	VIT_14s00 30g00710	VIT_15s00 24g00280	VIT_19s00 90g01480	VIT_01s00 11g06550
ARRIAT	12	8	12	8	10	10	11
CAATBOX1	3	14	9	6	18	12	13
CACTFTPPCA1	25	14	16	14	20	19	10
DOFCOREZM	14	24	13	15	22	22	19
GATABOX	7	5	4	5	11	12	4
GT1GMSCAM4	7	4	3	1	3	2	3
MYCCONSENSUSAT	16	8	10	6	6	8	2
POLASIG1	2	11	1	11	8	2	1
POLASIG3	4	6	1	2	7	7	4
POLLENILELAT52	11	16	4	12	4	9	6
GTGANTG10	4	5	13	6	8	9	10
EBOXBNNAPA	16	8	10	6	6	6	2

In cases of multiple transcripts annotated for one gene locus, the longest one was chosen. Finally, the non-redundant genes were assigned as the *CPA* genes. Their nomenclature was based on that set forth by the International Grapevine Genome Program and implemented recently (Liu et al., 2014; Wang et al., 2014; Grimplet et al., 2014).

All grapevine genes have named after the nomenclature recommended by the International Grapevine Genome Program based on the locations of the genes in the chromosomes (Grimplet et al., 2014).

Phylogenetic analysis and classification of the *CPA* super-family

Multiple *CPA* sequences were aligned using ClustalX2 program. A NJ (Neighbor-Joining) tree was constructed according to the alignments with p-distance and 1,000 bootstrap repeats by using ClustalW (<http://align.genome.jp/clustalw/>). All identified *CPA* genes were classified into different groups based on the *AtCPA* classification.

Gene structures and conserved domains

Gene structure information was collected from Phytozome database, the TAIR10 Genome Release, and the CRIBI database. The conserved domain data were analyzed using SMART (<http://smart.embl-heidelberg.de>) (Zhang et al., 2012c) and Pfam. The exon/intron organization for the *CPA1* genes was illustrated by software fancyGENE (<http://bio.ieu.eu/fancygene/>) (Tang et al., 2008b). We use the MEME tool (<http://meme.nbcr.net/meme/intro.html>) to analysis the motif for the *CPA1* genes of grape and *Arabidopsis*. MEME program was performed with motif length set as 10 to 200, maximum number of motifs 50.

Mapping grapevine *CPA* genes on chromosomes

Grapevine *CPA* genes were positioned onto grapevine chromosomes based on the V1 whole genome annotation from Grapevine genome CRIBI website (<http://genomes.cribi.unipd.it/>). The map was drafted with the MapInspect software (<http://www.plantbreeding.wur.nl/uk/software-mapinspect.html>). Tandem duplications of *CPA* genes in the grapevine genome were identified by checking their physical locations within a 100-kb adjacent region in individual chromosomes (Yang et al., 2008). The information for synteny blocks within the grapevine genome and between grapevine and *Arabidopsis* genomes were obtained from Plant Genome Duplication Database (<http://chibba.agtec.uga.edu/>) (Tang et al., 2008a).

Conclusion

In this research, we identified 29 genes in the *CPA* super-family in the grapevine reference genome and classified them into two groups and five subgroups. The number of *CPA* genes in this study differed from previous studies partially because we used the more updated grapevine V1 database in a community-designated website, rather than an older version (www.Phytozome.net) used in the previous studies. In addition, we used the V1 annotation from Grapevine genome CRIBI website (<http://genomes.cribi.unipd.it/>), which has been regularly updated. The protein domain structures we used for *CPA* classifications are more consistent with previously reported studies of functional characterizations on *CPA* genes. Over 50% (15, 57.72%) of *VvCPAs* were found to be associated with duplication events, inferred that the gene duplication plays an important role in evolutionary history of grapevine *CPAs*. This comprehensive information of the grapevine *CPA* gene family lays a foundation for further functional characterization of this gene family in grapevine salt tolerance.

Acknowledgment

This project was funded by the Priority Academic Program Development of Modern Horticultural Science in Jiangsu Province and Chinese Ministry of Agriculture 948 project.

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