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# Plant ascorbate peroxidase: molecular phylogeny and role in oxidative stress

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**SUMMARY.** Oxidative stress appears as a condition in accumulation and detoxification of reactive oxygen species (ROS). ROS are oxygen-derived free radicals, generated predominantly in mitochondria, peroxisomes and chloroplasts, as natural byproducts of the normal cell aerobic metabolism. In spite of their damaging effect, ROS can act as secondary messengers in different cellular processes, including tolerance to environmental stress factors. To neutralize the harmful effects of ROS, plants have evolved enzymatic and non-enzymatic defense systems. In flowering plants, ascorbate peroxidase (APX) is present in eight isoenzyme forms and constitutes an important enzymatic component in scavenging the harmful hydrogen peroxide to water as part of ascorbate-glutathione cycle. APX proteins, their roles, *in planta* expression location and their phylogenetic relationships are presented in the current paper. The phylogenetic analysis performed with the maximum likelihood method which was established for 118 protein sequences of 45 flowering plants. Our phylogenetic analysis revealed diversification of ascorbate peroxidase in angiosperms, and indicates a close relationship of APX1 with APX2, APX3 with APX4 and APX5, and APX6 with sAPX and tAPX proteins. Evolutionary relationships of plant ascorbate peroxidase isoenzymes indicate the evolution of different plant species genome and their phylogenetic affiliation.

**Keywords:** antioxidative defense system, evolution, osmoprotectants, phylogenetic

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## Introduction

Over the course of their lifetime, plants are exposed to different adverse environmental conditions, like osmotic and oxidative stress. In order to protect themselves against harsh external conditions, plants accumulate a series of protecting compounds, called osmoprotectants, and activate their antioxidative defense system. Appearance of $O_2$-evolving photosynthetic organisms and aerobic metabolism inevitably generated the occurrence of highly reactive oxygen species (ROS) (Halliwell 2006). During photosynthesis, oxygen is generated in the chloroplasts, and can accept electrons, thus forming $O_2^{\cdot -}$ (superoxide radical). In a multistep reaction, different types of ROS are generated from ground state oxygen i.e. $O_2^{\cdot -}$ (superoxide radical) and leads to the formation of $H_2O_2$ (hydrogen peroxide), $^1O_2$ (singlet oxygen), $HO_2^{\cdot}$ (perhydroxy radical), $HO^{\cdot}$ (hydroxyl radical), ROOH (alkyl hydroperoxide radical), $ROO^{\cdot}$ (alkylperoxyl radical) and $RO^{\cdot}$ (alkoxyl radical), which are highly reactive molecules causing serious damage to cell components and DNA, conducting to cell death (Gill and Tuteja, 2010). Under steady state conditions, damaging ROS molecules are scavenged by a set of antioxidative defense systems characteristic to chloroplasts, mitochondria and peroxisomes (Foyer and Harbinson, 1994; Alscher *et al.,* 1997; Klotz, 2002; Apel and Hirt, 2004; Navrot *et al.,* 2007; Heyno *et al.,* 2011; Sharma *et al.,* 2012), thus maintaining equilibrium between production and scavenging of ROS. This equilibrium can be disturbed by various biotic and abiotic environmental stress factors, such as pathogen attacks, salinity, drought, extreme temperatures, intense light, heavy metals, air pollution, herbicides and mechanical stress. Due to adverse stress factors, the levels of ROS in cells can suddenly increase and cause serious cell structure damages (Elstner, 1991; Malan *et al.,* 1990; Tsugane *et al.,* 1999).

Plant antioxidative defense mechanisms are of two types: enzymatic and non-enzymatic. Enzymatic system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and enzymes of ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Non-enzymatic system comprises of ascorbate (AsA), glutathione (GSH), tocopherols, carotenoids, and flavonoids (Noctor and Foyer, 1998; Sharma *et al.,* 2012) (Fig. 1).

It is important to mention that the osmoprotectant molecule, proline, can act as a non-enzymatic antioxidant needed to counteract the damaging effects of ROS in organisms as microbes, plants and animals (Chen and Dickman, 2005; Székely *et al.,* 2008). Despite their deleterious effect, ROS can act as secondary messengers in different cellular processes, including tolerance to environmental stresses (Desikan *et al.,* 2001; Yan *et al.,* 2007; Sharma *et al.,* 2012). The aim of this article is to present the plant ascorbate peroxidases (APX) (EC 1.11.1.11) phylogeny, as a main participant in neutralizing ROS.

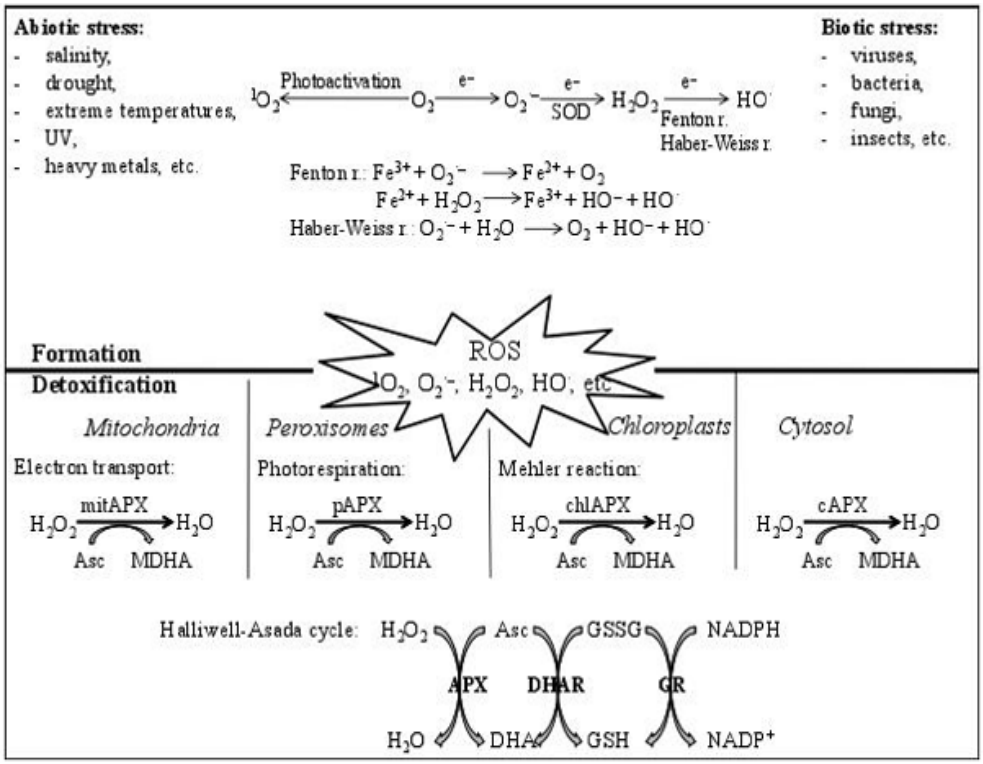


**Figure 1.** Schematic representation of APX role in ROS formation and detoxification in plants; ROS formation: $^1O_2$: singlet oxygen, $O_2$: molecular oxygen, $O_2^{\bullet-}$: superoxide radical, SOD: superoxide dismutase, $H_2O_2$: hydrogen peroxide, $HO^{\bullet}$: hydroxyl radical. ROS detoxification: Asc: ascorbate, MDHA: monodehydroascorbate; Halliwell-Asada cycle: $H_2O_2$: hydrogen peroxide, APX: ascorbate peroxidase, $H_2O$: water, DHAR: dehydroascorbate reductase, DHA: dehydroascorbate, GSSG: oxidized glutathione, GR: glutathione reductase, GSH: reduced glutathione, NADPH: reduced nicotinamide adenine dinucleotide phosphate, $NADP^+$: nicotinamide adenine dinucleotide phosphate (based on Noctor and Foyer, 1998; Sharma *et al.,* 2012).

### Role of APX enzyme in antioxidative defense system during stress conditions

In plants, APX is the most distributed antioxidant enzyme and is considered to be a key ROS scavenger enzyme and cell protecting molecule (Orvar and Ellis, 1997). APX, together with catalase, controls the level of $H_2O_2$ in cells, but the main $H_2O_2$ scavenging role is thought to belong to APX, which converts $H_2O_2$ to $H_2O$ in water-water and AsA-GSH (ascorbate glutathione or Halliwell Asada) cycles. APX uses two molecules of AsA to reduce $H_2O_2$ to water with subsequent generation of two molecules of MDHA (monodehydroascorbate) (Fig. 1).

Based on amino acid sequences, several different isoforms of APX family have been found at different subcellular localization in flowering plants, including chloroplast, mitochondria, peroxisomes and cytosol (Jimenez *et al.,* 1997; Madhusudhan *et al.,* 2003; Sharma and Dubey, 2004; Nakano and Asada, 1987). The organelle APX is efficient in scavenging $H_2O_2$ produced in the organelles, while cytosolic APX neutralizes $H_2O_2$ from cytosol, apoplast and that diffused from organelles (Sharma *et al.,* 2012). APX isoforms have a much higher affinity for $H_2O_2$ compared to CAT and are essential in scavenging ROS during stress conditions (Wang *et al.,* 1999). Many publications reported enhanced level of APX enzyme activity during different abiotic stress conditions, such as salinity, drought, extreme temperatures, heavy metal toxicity and presence of high-light intensities (Boo and Jung, 1999; Sharma and Dubey, 2005a; Sharma and Dubey, 2007; Han *et al.,* 2009; Maheshwari and Dubey, 2009; Hefny and Abdel-Kader, 2009). Begara-Morales *et al.,* (2013) reported the increase of APX enzyme activity in pea plants grown under saline (150 mM NaCl) conditions, *Anabaena doliolum* also revealed enhanced APX activity during salt stress (Srivastava *et al.,* 2005), water stress induced APX activity in three cultivars of *Phaseolus vulgaris* (Zlatev *et al.,* 2006) and *P. asperata* (Yang *et al.,* 2008), mild drought stress in rice generated higher chloroplastic-APX activity (Sharma and Dubey, 2005b), Cd stress caused increased APX activity in leaves of *Ceratophyllum demersum* (Arvind and Prasad, 2003), *Brassica juncea* (Mobin and Chan, 2007), *Triticum aestivum* (Khan *et al.,* 2007) and *Vigna mungo* (Singh *et al.,* 2008). Simonovicova *et al.,* (2004) reported enhancement of APX activity in *Hordeum vulgare* roots in the presence of Al stress. However, biochemical methods currently used to assess the enzyme APX activity can evaluate only the total APX activity, without distinguishing between the activities of the different APX isoforms.

## Materials and methods

### Data collection

The ascorbate peroxidase protein sequences used in this paper were gathered from the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) (McEntyre and Ostell, 2002) database and from arabidopsis.org. The sequences were run through BLAST Sequence Analysis Tool online program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in a non-redundant database (McEntyre and Ostell, 2002).

### Sequence similarity

APX sequence similarity between *Arabidopsis thaliana* and the studied species was calculated using matGAT software with first gap penalty 12 and expanding gap 2 settings. (Campanella *et al.,* 2003). We collected separately the monocots and dicots APX sequences, the sequences which did not correspond to

homology were not taken into account. Thus, the similarity score was obtained from comparing 118 protein sequence. The scoring matrix used was BLOSUM50, penalties for first gap 16, and extending gap 4. The query monocots sequences were assessed comparing their similarity with *Arabidopsis thaliana* APXs (reference sequences: P82281, Q05431, Q1PER6, Q42564, Q42592, Q42593, Q7XZP5, Q8GY91). The predicted subcellular localization was obtained from http://cello.life.nctu.edu.tw/ (Yu *et al.,* 2004; Yu *et al.,* 2006) with the specific parameters ("Eukaryotes" and "Protein").

**Magnoliophyta APX proteins**

APX protein forms of 45 species from Magnoliophyta phylum were analyzed and compared to each other. The protein sequences were classified based on Catalogue of Life database (Table 2). Magnoliopsida class analysis indicated several families: Brassicaceae and Capparaceae family (APX1-3 and APX5); Capparaceae and Chenopodiaceae family (APX1-3 and APX6); Cucurbitaceae family (APX1,2 and APX6); Euphorbiaceae family (APX1, APX3, APX6); Fabaceae family (APX2) Malvaceae (APX1-2); Moraceae (APX1), Nelumbonaceae (APX2); Pedaliaceae and Rosaceae family (APX1-3, APX6), Rutaceae (APX1-3); Salicaceae (APX1-2, APX6); Solanaceae (APX1, APX3, APX6), Sterculiaceae family (APX2); Vitaceae family (APX1-2, APX6). From Liliopsida class Poaceae family (APX1-2, APX4, APX6, sAPX, tAPX) was analyzed.

**Classification of APX isoforms**

In order to fulfil an accurate analysis, a table containing the eight *Arabidopsis thaliana* AtAPX isoforms was created, as a benchmark for all APX proteins of the examined species (Table 1). The APX protein sequences were grouped according to their similarity index. Table 1 shows the APX isoforms according to NCBI database and the same isoforms based on their sequence alignments. The highest APX similarity values were used in our analyses. Similarity values below 50% were not considered.

**Phylogenetic tree**

For phylogenetic analyses 118 sequences were used: APX1 (25 sequences), APX2 (26 sequences), APX3 (10 sequences), APX4 (11 sequences), APX5 (11 sequences), APX6 (15 sequences), sAPX (10 sequences), tAPX (10 sequences) (Table 1). The protein sequences were sorted and aligned by multiple sequence alignments with ClustalW in MEGA7 program (Kumar *et al.,* 2016) using default settings. The length of aligned protein sequences were 271 amino acids. The phylogenetic tree was generated in PhyML SMS (Guindon *et al.,* 2010; Lefort *et al.,* 2017) maximum likelihood framework program. The phylogenetic reconstruction

was performed assuming a LG +G+I (Le and Gascuel, 2008) evolution model with gamma distributed variation rate across site (G) and a proportion of invariable site (I). The statistics value was based on Shimodaira–Hasegawa [SH] approximate likelihood ratio test [aLRT]. Phylogenetic tree was created by FigTree (v 1.4.0.) program. Main genetic distance between groups and within groups was estimated using p-distance (bootstrap value 1000) by MEGA7 program.

## Results and discussion

Table 1 summarizes the APX isoforms, their cellular localization, role and expression location in *A. thaliana*.

**Table 1.**

APX isoforms and their role in *Arabidopsis thaliana* according to arabidopsis.org

| APX protein | TAIR accession no. | Cellular localization | Role induced by | *In-planta* expression location |
|---|---|---|---|---|
| APX1 | At1g07890 | Golgi apparatus, chloroplast stroma, cytoplasm, plasma membrane, plasmodesma, cell wall | Cd ion, cytokinin, heat, oxidative stress, reactive oxygen species, salt stress | root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen, pollen tube cell,) fruit, seed, guard cell |
| APX2 | At3g09640 | cytoplasm | hydrogen peroxide catabolic process, oxidation-reduction process, oxidative stress | leaf |
| APX3 | At4g35000 | chloroplast envelope, glyoxysomal membrane, mitochondrion, peroxisomal membrane, plasmodesma, plastid, vacuolar membrane | hydrogen peroxide catabolic process, oxidation-reduction process, cytokinin, oxidative stress | root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen, pollen tube cell,) seed, guard cell |

| APX protein | TAIR accession no. | Cellular localization | Role induced by | *In-planta* expression location |
|---|---|---|---|---|
| APX4 | At4g09010 | chloroplast thylakoid lumen, chloroplast thylakoid membrane, cytoplasm, nucleus | oxidation-reduction process, oxidative stress | shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell |
| APX5 | At4g35970 | integral component of peroxisomal membrane | oxidative stress, hydrogen peroxide removal, hydrogen peroxide catabolic process, oxidation-reduction process | root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell |
| APX6 | At4g32320 | cytosol, extracellular region | hydrogen peroxide catabolic process, oxidative stress, seed germination, seed maturation | root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell |
| sAPX | At4g08390 | chloroplast stroma, mitochondrion membrane | hydrogen peroxide catabolic process, oxidation-reduction process, cytokinin, oxidative stress | root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell |
| tAPX | At1g77490 | integral component of chloroplast thylakoid membrane | chloroplast-nucleus signaling pathway, cold acclimation, hydrogen peroxide catabolic process, hydrogen peroxide mediated signaling pathway, oxidation-reduction process, oxidative stress | root, shoot, leaf, flower (flower pedicel, sepal, petal, stamen, pollen) seed, guard cell |

Plant species and their APX isoforms are visible in figures representing the phylogenetic tree of APX proteins. Table 2 shows the cellular localization of the studied 45 plant species.

**Table 2.**

List of analyzed species, APX isoforms and their subcellular localization

| | **Species name** | **NCBI GenBank ID** | **APX** | **Subcellular localization** |
|---|---|---|---|---|
| 1 | *Arabidopsis thaliana* | AEE28201.1 | APX1 | Cytoplasmic |
| 2 | *Arabidopsis thaliana* | Q05431.2 | APX1 | Cytoplasmic |
| 3 | *Beta vulgaris* subsp. *vulgaris* | XP_010691257.1 | APX1 | Cytoplasmic |
| 4 | *Brachypodium distachyon* | XP_003558178.1 | APX1 | Cytoplasmic |
| 5 | *Brassica rapa* | XP_009118405.1 | APX1 | Cytoplasmic |
| 6 | *Citrus maxima* | ACM17464.1 | APX1 | Cytoplasmic |
| 7 | *Cucumis sativus* | AGJ72850.1 | APX1 | Cytoplasmic |
| 8 | *Gossypium hirsutum* | ABR18607.1 | APX1 | Cytoplasmic |
| 9 | *Jatropha curcas* | ACV50426.1 | APX1 | Cytoplasmic |
| 10 | *Malus domestica* | ABP87792.1 | APX1 | Cytoplasmic |
| 11 | *Morus notabilis* | EXC33221.1 | APX1 | Cytoplasmic |
| 12 | *Nicotiana sylvestris* | XP_009784425.1 | APX1 | Cytoplasmic |
| 13 | *Nicotiana tabacum* | AAA86689.1 | APX1 | Cytoplasmic |
| 14 | *Nicotiana tomentosiformis* | XP_009597491.1 | APX1 | Cytoplasmic |
| 15 | *Oryza brachyantha* | XP_006649890.1 | APX1 | Cytoplasmic |
| 16 | *Oryza sativa Japonica* | ABF95353.1 | APX1 | Cytoplasmic |
| 17 | *Oryza sativa Japonica* | Q10N21.1 | APX1 | Cytoplasmic |
| 18 | *Populus euphratica* | XP_011008849.1 | APX1 | Cytoplasmic |
| 19 | *Prunus mume* | XP_008224940.1 | APX1 | Cytoplasmic |
| 20 | *Sesamum indicum* | XP_011089855.1 | APX1 | Cytoplasmic |
| 21 | *Setaria italica* | XP_004984819.1 | APX1 | Cytoplasmic |
| 22 | *Solanum lycopersicum* | AAZ77770.1 | APX1 | Cytoplasmic |
| 23 | *Solanum tuberosum* | NP_001275066.1 | APX1 | Cytoplasmic |
| 24 | *Spinacia oleracea* | BAA12890.1 | APX1 | Cytoplasmic |
| 25 | *Tarenaya hassleriana* | XP_010521780.1 | APX1 | Cytoplasmic |
| 26 | *Vitis vinifera* | NP_001267988.1 | APX1 | Cytoplasmic |
| 27 | *Zea mays* | NP_001152249.1 | APX1 | Cytoplasmic |
| 28 | *Aegilops tauschii* | EMT09178.1 | APX2 | Cytoplasmic |
| 29 | *Arabidopsis thaliana* | AEE74792.1 | APX2 | Cytoplasmic |
| 30 | *Arabidopsis thaliana* | Q1PER6.3 | APX2 | Cytoplasmic |
| 31 | *Beta vulgaris* subsp. *vulgaris* | XP_010696372.1 | APX2 | Cytoplasmic |
| 32 | *Brachypodium distachyon* | XP_003562395.1 | APX2 | Cytoplasmic |
| 33 | *Brassica rapa* | XP_009123280.2 | APX2 | Cytoplasmic |
| 34 | *Brassica rapa* subsp. *oleifera* | CCC55736.1 | APX2 | Cytoplasmic |
| 35 | *Camelina sativa* | XP_010486536.1 | APX2 | Cytoplasmic |
| 36 | *Citrus maxima* | ACM17463.1 | APX2 | Cytoplasmic |
| 37 | *Citrus sinensis* | XP_006480586.1 | APX2 | Cytoplasmic |
| 38 | *Fragaria vesca* subsp. *vesca* | XP_004302839.1 | APX2 | Cytoplasmic |
| 39 | *Glycine max* | AAB01221.1 | APX2 | Cytoplasmic |
| 40 | *Gossypium arboreum* | KHG05754.1 | APX2 | Cytoplasmic |
| 41 | *Malus domestica* | XP_008350397.1 | APX2 | Cytoplasmic |

| | Species name | NCBI GenBank ID | APX | Subcellular localization |
|---|---|---|---|---|
| 42 | *Momordica charantia* | AIE12238.1 | APX2 | Cytoplasmic |
| 43 | *Nelumbo nucifera* | XP_010253495.1 | APX2 | Cytoplasmic |
| 44 | *Oryza brachyantha* | XP_006658179.1 | APX2 | Cytoplasmic |
| 45 | *Oryza sativa Japonica* | Q9FE01.1 | APX2 | Cytoplasmic |
| 46 | *Populus euphratica* | XP_011048406.1 | APX2 | Cytoplasmic |
| 47 | *Prunus mume* | XP_008239139.1 | APX2 | Cytoplasmic |
| 48 | *Sesamum indicum* | XP_011094725.1 | APX2 | Cytoplasmic |
| 49 | *Setaria italica* | XP_004958804.1 | APX2 | Cytoplasmic |
| 50 | *Tarenaya hassleriana* | XP_010543364.1 | APX2 | Cytoplasmic |
| 51 | *Theobroma cacao* | EOY07733.1 | APX2 | Cytoplasmic |
| 52 | *Theobroma cacao* | XP_007016653.2 | APX2 | Cytoplasmic |
| 53 | *Vitis vinifera* | XP_010651099.1 | APX2 | Cytoplasmic |
| 54 | *Zea mays* | NP_001105500.2 | APX2 | Cytoplasmic |
| 55 | *Arabidopsis lyrata* subsp. *lyrata* | XP_020874660.1 | APX3 | Cytoplasmic |
| 56 | *Arabidopsis thaliana* | Q42564.1 | APX3 | Cytoplasmic |
| 57 | *Beta vulgaris* subsp. *vulgaris* | XP_010679905.1 | APX3 | Mitochondrial |
| 58 | *Brassica rapa* | XP_009138334.1 | APX3 | Cytoplasmic |
| 59 | *Citrus sinensis* | XP_006486751.1 | APX3 | Cytoplasmic |
| 60 | *Nicotiana tomentosiformis* | XP_009618135.1 | APX3 | Cytoplasmic |
| 61 | *Oryza sativa Japonica* | Q0JEQ2.1 | APX3 | Cytoplasmic |
| 62 | *Ricinus communis* | XP_002530823.1 | APX3 | Cytoplasmic |
| 63 | *Sesamum indicum* | XP_011088597.1 | APX3 | Cytoplasmic |
| 64 | *Solanum tuberosum* | XP_006359692.1 | APX3 | Cytoplasmic |
| 65 | *Vitis vinifera* | XP_002278281.1 | APX3 | Cytoplasmic |
| 66 | *Aegilops tauschii* | EMT10887.1 | APX4 | Cytoplasmic |
| 67 | *Aegilops tauschii* subsp. *tauschii* | XP_020163634.1 | APX4 | Cytoplasmic |
| 68 | *Arabidopsis thaliana* | P82281.2 | APX4 | Chloroplast |
| 69 | *Brachypodium distachyon* | XP_003574893.1 | APX4 | Cytoplasmic |
| 70 | *Hordeum vulgare* subsp. *vulgare* | BAB62533.1 | APX4 | Cytoplasmic |
| 71 | *Oryza brachyantha* | XP_006659666.1 | APX4 | Chloroplast |
| 72 | *Oryza sativa Japonica* | Q6ZJJ1.1 | APX4 | Chloroplast |
| 73 | *Oryza sativa Japonica* | XP_015650808.1 | APX4 | Chloroplast |
| 74 | *Setaria italica* | XP_004974146.1 | APX4 | Cytoplasmic |
| 75 | *Sorghum bicolor* | XP_002444620.1 | APX4 | Cytoplasmic |
| 76 | *Stipa purpurea* | AJF34885.1 | APX4 | Cytoplasmic |
| 77 | *Zea mays* | NP_001132505.1 | APX4 | Cytoplasmic |
| 78 | *Arabidopsis lyrata* subsp. *lyrata* | XP_002869048.1 | APX5 | Cytoplasmic |
| 79 | *Arabidopsis thaliana* | Q7XZP5.2 | APX5 | Cytoplasmic |
| 80 | *Arabidopsis thaliana* | NP_195321.1 | APX5 | Cytoplasmic |
| 81 | *Arabidopsis thaliana* | AAP72144.1 | APX5 | Cytoplasmic |
| 82 | *Brassica napus* | XP_013743324.1 | APX5 | Cytoplasmic |
| 83 | *Brassica rapa* | XP_009148286.2 | APX5 | Cytoplasmic |
| 84 | *Camelina sativa* | XP_010432206.1 | APX5 | Cytoplasmic |
| 85 | *Camelina sativa* | XP_010446847.1 | APX5 | Cytoplasmic |

| | Species name | NCBI GenBank ID | APX | Subcellular localization |
|---|---|---|---|---|
| 86 | *Capsella rubella* | XP_006285911.1 | APX5 | Nuclear |
| 87 | *Eutrema salsugineum* | XP_006412038.1 | APX5 | Nuclear |
| 88 | *Oryza sativa Japonica* | P0C0L0.1 | APX5 | Chloroplast |
| 89 | *Tarenaya hassleriana* | XP_010526807.1 | APX5 | Cytoplasmic |
| 90 | *Arabidopsis thaliana* | Q8GY91.1 | APX6 | Chloroplast |
| 91 | *Beta vulgaris* subsp. *vulgaris* | XP_010674956.1 | APX6 | Chloroplast |
| 92 | *Brachypodium distachyon* | XP_003578843.1 | APX6 | Mitochondrial |
| 93 | *Cucumis sativus* | XP_004149799.1 | APX6 | Chloroplast |
| 94 | *Fragaria vesca* subsp. *vesca* | XP_004290885.1 | APX6 | Chloroplast |
| 95 | *Jatropha curcas* | XP_012078304.1 | APX6 | Chloroplast |
| 96 | *Malus domestica* | XP_008365199.2 | APX6 | Chloroplast |
| 97 | *Nicotiana sylvestris* | XP_009784836.1 | APX6 | Chloroplast |
| 98 | *Nicotiana tomentosiformis* | XP_009629390.1 | APX6 | Chloroplast |
| 99 | *Oryza sativa Japonica* | P0C0L1.1 | APX6 | Mitochondrial |
| 100 | *Oryza sativa Japonica* | ABA96617.1 | APX6 | Mitochondrial |
| 101 | *Populus euphratica* | XP_010999402.1 | APX6 | Chloroplast |
| 102 | *Prunus mume* | XP_008219620.1 | APX6 | Chloroplast |
| 103 | *Sesamum indicum* | XP_011074839.1 | APX6 | Chloroplast |
| 104 | *Setaria italica* | XP_004977222.1 | APX6 | Mitochondrial |
| 105 | *Solanum lycopersicum* | NP_001234631.2 | APX6 | Chloroplast |
| 106 | *Vitis vinifera* | XP_003634424.1 | APX6 | Chloroplast |
| 107 | *Aegilops tauschii* subsp. *tauschii* | XP_020162413.1 | sAPX | Chloroplast |
| 108 | *Arabidopsis thaliana* | Q42592.2 | sAPX | Chloroplast |
| 109 | *Brachypodium distachyon* | XP_003579783.1 | sAPX | Chloroplast |
| 110 | *Hordeum vulgare* subsp. *vulgare* | BAJ97403.1 | sAPX | Chloroplast |
| 111 | *Oryza brachyantha* | XP_006652305.1 | sAPX | Chloroplast |
| 112 | *Oryza sativa* | CAH67301.1 | sAPX | Chloroplast |
| 113 | *Oryza sativa Indica Group* | EEC77311.1 | sAPX | Chloroplast |
| 114 | *Oryza sativa Japonica* | Q7XJ02.1 | sAPX | Chloroplast |
| 115 | *Oryza sativa Japonica* | XP_015635863.1 | sAPX | Chloroplast |
| 116 | *Setaria italica* | XP_004975656.1 | sAPX | Mitochondrial |
| 117 | *Sorghum bicolor* | XP_021319656.1 | sAPX | Chloroplast |
| 118 | *Aegilops tauschii* subsp. *tauschii* | XP_020185648.1 | tAPX | Chloroplast |
| 119 | *Arabidopsis thaliana* | Q42593.2 | tAPX | Chloroplast |
| 120 | *Brachypodium distachyon* | XP_010235542.1 | tAPX | Chloroplast |
| 121 | *Oryza brachyantha* | XP_006647364.1 | tAPX | Chloroplast |
| 122 | *Oryza sativa Japonica* | Q69SV0.2 | tAPX | Chloroplast |
| 123 | *Saccharum hybrid cultivar* | AGD80597.1 | tAPX | Chloroplast |
| 124 | *Setaria italica* | XP_004952823.1 | tAPX | Chloroplast |
| 125 | *Sorghum bicolor* | XP_021316117.1 | tAPX | Chloroplast |
| 126 | *Triticum aestivum* | AAS80158.1 | tAPX | Chloroplast |
| 127 | *Triticum urartu* | EMS46926.1 | tAPX | Chloroplast |

Phylogeny of APX isoforms out of 45 flowering plant species is represented by Figure 2.

The APX isoforms constitute a monophyletic group. The APX phylogenetic tree has split three branches with highly supported values (1 SH). Red band indicates the phylogenesis of APX1 and APX2 proteins, with highest number of species. These groups APX1 and APX2 sequences are mixed. The first lineage (0.677 SH-like support value) consists of species like Vitaceae, Pedaliaceae, Brassicaceae, Capparaceae, Malvaceae, Chenopodiaceae, Rutaceae, Rosaceae, Salicaceae families. Rutaceae family members has sister groups with Chenopodiaceae (APX2), Malvaceae (APX2), Capparaceae (APX2) and Brassicaceae (APX2) family species. The next lineage, Liliopsida class, consists of Poaceae family with APX1-2 groups, and they are sister groups (0.872 SH). Inside the Poaceae family there is a split between APX1 and APX2. The next split is the Malvales class with the highest confidence interval (0.780 SH). In close relation (0.8 SH) appears the Rosaceae with Moraceae and Cucurbitaceae family species. These groups are sister groups (0.826 SH) with Chenopodiaceae, Solanaceae, Brassicaceae, Capparaceae, Pedaliaceae family species. The second big branching are APX3, 4, 5 groups (1SH). The light green band shows the APX3 as a well-supported clade (0.895 SH) with three divergences: Chenopodiaceae, Brassicaceae and the close related Solanaceae, Vitaceae, Euphorbiaceae, Rutaceae, Pedaliaceae (0.895 SH) families. Another well supported clade is the APX4 marked with dark blue band, which comprise Poaceae family species and is separated in two sister groups with the highest divergence (1 SH). A well separated group is the APX5, colored with light green band, and consists the Capparaceae family and a highly separated Brassicaceae family species (0.998 SH). The last big branching is APX6, sAPX and tAPX groups (1 SH). APX6 is marked with light blue and is a well separated clade. The first clade is Liliopsida class (0.97 SH) with a well-supported clade, Poaceae family members (0.966 SH). The next branch is composed of Chenopodiaceae, Euphorbiaceae, Vitaceae, Rosaceae, Salicaceae, Pedaliaceae, Solanaceae, Cucurbitaceae families. The Vitaceae and Cucurbitaceae generates a sibling group and are consisted of one sister group, the Rosaceae family species. sAPX appears as a well-supported clade marked with lilac band. sAPX of *Oryza* genus are weakly supported. The yellow tAPX are well isolated group (1 SH), with two sister clades: *Oryza* genus and the Poaceae family species (0.375 SH). However, the low support of nodes on the phylogenetic tree shows that the relationship between the species is not clearly resolved yet. The high level of differentiation of APX groups is also confirmed by the high p-distance value expressed as a percentage value and appears as a high level of divergence between groups (Table 3).

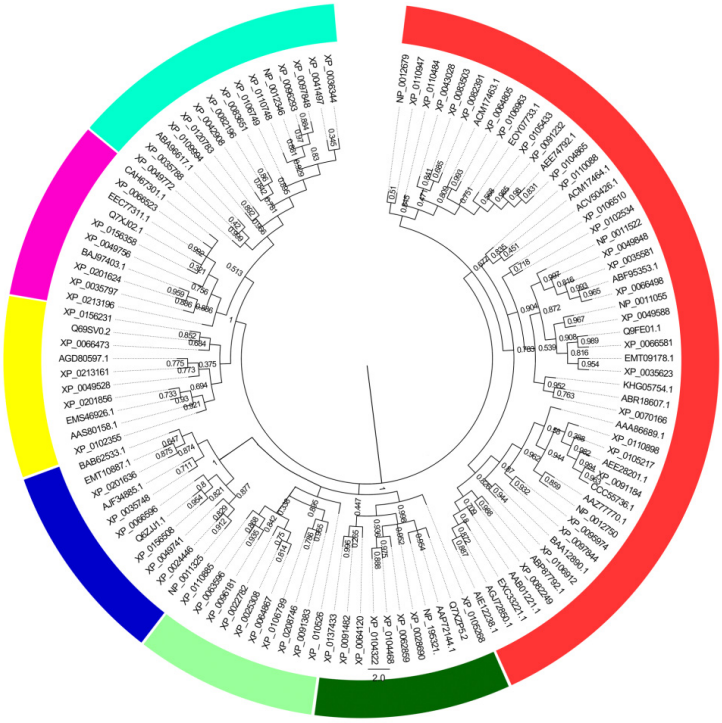

**Figure 2.** PhyML phylogenetic tree of 45 flowering plant species APX proteins; APX1 and APX2 are marked with red band, APX3 with light green band, APX4 with dark blue band, APX5 with light green band, APX6 with light blue band, sAPX with lilac band and tAPX with yellow band.

**Table 3.**

Average genetic distance between APX groups (%)

| Groups name | APX1 | APX2 | APX3 | APX4 | APX5 | APX6 | sAPX |
|---|---|---|---|---|---|---|---|
| APX2 | 16.7 | | | | | | |
| APX3 | 33.1 | 33.2 | | | | | |
| APX4 | 33.6 | 33.4 | 19.1 | | | | |
| APX5 | 38.7 | 38.3 | 26.8 | 31.8 | | | |
| APX6 | 50.7 | 50.6 | 46.2 | 44.9 | 51.7 | | |
| sAPX | 49.6 | 49.3 | 45.5 | 45.3 | 50.5 | 16.4 | |
| tAPX | 50.3 | 50.4 | 46.3 | 45.5 | 52.4 | 16.0 | 12.2 |

The genetic distance analyses within groups were also analyzed. The groups APX1-2-3 and APX6 revealed the higher genetic distance, exceeding the value of 1.1% (Table 4).

**Table 4.**

Average genetic distance within the APX groups (%)

| Groups name | Genetic distance |
|---|---|
| APX1 | 1.59 |
| APX2 | 1.54 |
| APX3 | 1.19 |
| APX4 | 0.66 |
| APX5 | 0.90 |
| APX6 | 1.26 |
| sAPX | 0.58 |
| tAPX | 0.55 |

Our cellular localization analysis indicated that APX1 and APX2 are localized in cytoplasm, APX3, APX4 and APX5 are localized in mitochondria, chloroplasts, nucleus and cytoplasm, APX6, sAPX and tAPX are mitochondrial and chloroplastic. The result of phylogeny reconstruction shows the relationship between the APX isoforms which may be influenced by the cellular localization also.

## Conclusions

Ascorbate peroxidase is an essential enzyme in detoxifying the extremely harmful hydrogen peroxide, therefore in plant oxidative stress response. Molecular phylogeny analysis of several plant APX proteins has been presented in this study, in order to investigate the molecular manner of evolution of ascorbate peroxidase isoenzymes family in angiosperms. Evolutionary analysis of ascorbate peroxidase isoenzymes of different plant species, showed that APX is a monophyletic group, probably evolved from a single ancestor, where some isoformes are close related, some are not. Our phylogenetic analysis points close relationships between APX1 and APX2, between APX3, APX4 and APX5, and between APX6, sAPX and tAPX proteins in angiosperms. With an essential role in detoxifying processes of the cell, understanding molecular mechanisms of stress tolerance and phylogeny of APX, can serve to generate an efficient drug for prevention of several diseases in plants, animals and humans caused by severely harmful oxidative stress. Gathering information about molecular function of APX isoforms to scavenge ROS in different cellular compartments, could contribute to stress responsive gene engineering in order to improve crop tolerance against adverse environmental conditions.

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