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RESEARCH ARTICLE

Genetics and metabolism of salicylic acid in plants

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Abstract – Genetics, biosynthesis, and metabolism of salicylic acid (SA) are analyzed here based on genbank data mining *in silico*. Amino acid compositions of plant *isochorismate synthase* (ICS) enzymes were found to be diverse; however, the sequence similarity dendrogram of the two ICS enzyme families of ICS1 and ICS2 showed significant differences aligned to those of *Arabidopsis thaliana* (Rédei, 1975) (*Eng.*, thale cress; *Hung.*, lúdfű). Biochemical routes of plant SA synthesis and metabolism are also analyzed here.

Keywords – biosynthesis, metabolism of salicylic acid, *Arabidopsis*

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INTRODUCTION

Salicylic acid (SA) is a simple organic molecule of plant metabolites that influences plant resistance to biotic and abiotic stresses and plant growth and development (Takagi *et al*., 2022).

SA in plants has a long history from the traditional medicinal use of the bark of white willow tree (*Salix alba*) (Fig. 1a) (*Hung.,* szomorúfűz; Simoncsics, 2017) described in the ancient books. Buchner, 1828, conducted the first chemical isolation of SA (Fig. 1b), as *salicin* (SAG) which is a storage form of SA (SA-β-glucoside; $C_{13}H_{18}O_7$; MW 286.28; Fig. 4), and naturally found in willow bark and other *Salicaceae* trees (Szabó and Botz, 1999). *Salicin* contents (% DW) showed high levels in *Salix* species. In *S. viminalis* it is ~5.0 %, in *S. americana* ~4.0 %, in *S. purpurea* ~4.0 %, in *S. alba* ~3.0 - 4.0 %, and in *S. fragilis* ~2.0 - 3.0 %. In general, twig tips harvested in winter showed the highest *salicin* content. Leaves also store *salicin*, especially in *S. purpurea* harvested in April of early spring (4.1%) (Szabó and Botz, 1999). *Vaccinium* species (*e.g., V. myrtillus*) (*Eng.*, European blueberry / myrtle; *Hung.*, fekete áfonya) also accumulate SA in dried leaves. Leaves of tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) also contain a considerable amount of endogenous SA (up to 20 µg/g FW) (Paterson *et al*., 2008; Klessig *et al*., 2016).

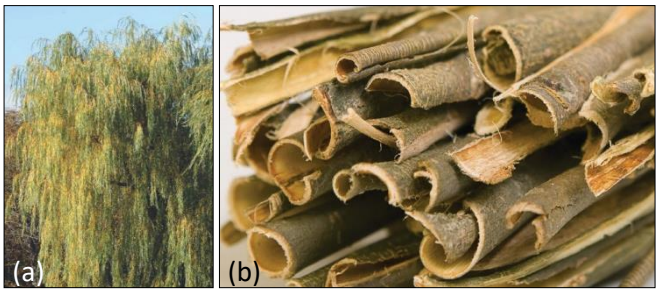

Figure 1a. White willow tree (*Salix alba*) (*Hung.,* szomorúfűz) *(a)*, and the harvested twig barks *(b)*, used for human pain and fever reliever (*web source*1).

SA was synthetically modified to acetylsalicylic acid (ASA) by Gerhardt (1853) and later manufactured by Bayer Co., Germany, as *aspirin* (1874), as an anti-inflammatory drug. Later, Ca-ASA was registered by Richter Co., Hungary, as *kalmopyrin* (1912); and as *istopyrin* (1933) registered by Biogal Co., Hungary, and all of them have been used for human medication (*In:* Raskin, 1992). SA dissolved in ethyl alcohol (2%) is also used to remove human skin acne. SA is

also used as traditional food conservation for canning fruits and jelly to save from mould (Noonim and Venkatachalam, 2022).

The application of SA and ASA for plant protection against diseases goes back to the works of Bernard (1911) and White (1979). Here we give an overview of the current state of SA research in plants.

BIOSYNTHESIS OF SA IN PLANTS

Both prokaryotes and eukaryotes can synthesize SA. In the human body SA is synthesized by bacteria living in the gastrointestinal tracts (Paterson *et al.*, 2008). In bacteria, *isochorismate pyruvate lyase* (IPL; UniProtKB 4.2.99.21; EC 4.2.99.21) catalyzes the reaction of *isochorismic acid* to SA (Torrens-Spence *et al.*, 2019) (Fig. 1b), however, IPL orthologues have not been identified in plants. In *Arabidopsis thaliana* (Rédei, 1975), exogenous SA is perceived by receptors of SA-binding proteins (SABPs), especially of NPR1 (P93002) and NPR3 (Q9SZI3) / NPR4 (Q5ICL9) proteins (NRP - Nonexpresser of PR [pathogen related] Regulatory Proteins). SABPs take part in the activation of pathways leading to enhanced disease resistance (Klessig *et al.*, 2016; Liu *et al.*, 2020).

Plants synthesize SA from *chorismic acid* in two routes (Fig.1b):
(1) via converting it to *isochorismate* (catalysed by ICS / MenF, EC 5.4.4.2.) followed by a conversion catalyzed by PBS3 enzyme (*4-substituted benzoates-glutamate ligase; Syn.: GH3.12 / GDG1 / PBS3 / WIN3 / AvrPphB: Susceptible*3) (the acronym comes from "AViRulance Protein ph*aseolus* B"). This enzyme (EC 6.3.2.; *At*GH3.12; TAIR: *At*5g13320) also catalyzes the conjugation of amino acids to 4-substituted benzoates (Tampakaki *et al.*, 2002; Okrent *et al.*, 2009); and EPS1 (*Enhanced Pseudomonas Susceptibility*1 enzyme; EC 2.3.1; TAIR: *At*5g67160) both enzymes functioning in the chloroplasts (Fig.1b) (Wildermuth *et al.*, 2001).
(2) Alternatively (Fig.1b), *chorismic acid* may be converted to *phenylalanine* (Qian *et al.*, 2019) *via* the PAL-enzyme pathway (*phenylalanine ammonia-lyase;* EC 4.3.1.24, 725 AA) (UniProtKB), and converted to SA, that may involve benzoic acid intermediates (Lefevere *et al.*, 2020). Mutant studies of *At*ICS2 gene suggest that its function is redundant to that of *At*ICS1 (*In:* NCBI). However, the double *Atics*1 and *Atics*2 mutant with blocked SA and Vitamin-K (*i.e.*, phylloquinone) synthesis has provided genetic evidence to an ICS-independent SA biosynthetic pathway in *Arabidopsis* (Garcion *et al.*, 2008).

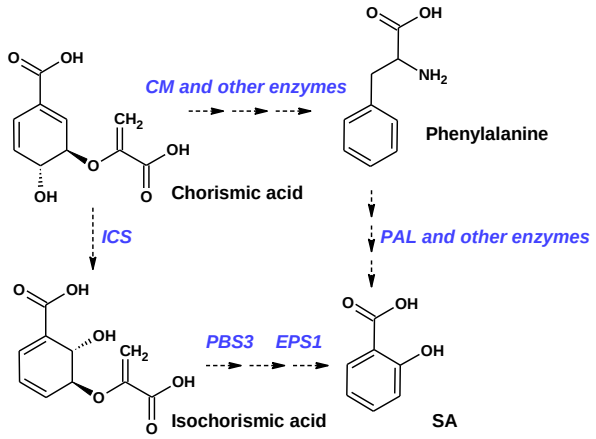

Figure 1b. The two main ways of SA (salicylic acid) biosynthesis in plants are initiated from *chorismic acid* (Fig. 3.) and *phenylalanine*. CM: *Chorismate mutase*, EC 5.4.99.5. ICS: *Isochorismate synthase*, EC 5.4.4.2. PBS3: *Substituted benzoates-glutamate ligase*, EC 6.3.2. EPS1: *Enhanced Pseudomonas Susceptibility*1 enzyme, EC 2.3.1. PAL *Phenylalanine ammonia-lyase*, EC 4.3.1.24.

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                                  430       440       450       460       470       480
                          ....|....|....|....|....|....|....|....|....|....|....|....|
NP_173321.4 ICS2[Arabidopsis t.] VRENIREKLKTICDRVVVKPHKSVRKLARVQHLYSQLAGQLKREDDEFNILTALHPTPAV
A0A6N2L7G9 ICS [Salix v.]         ......K..EAV...I..E.N.TI...PIL....AG...E.RS.........SS.......
XP_009104748.1 ICS1[Brassica r.] .........NS...K.....Q.T..................R.R......D..A........
XP_006302076.1 ICS1[Capsella r.] .........DG.........Q.T..................K.R......D..A........

                                  490       500       510       520       530       540
                          ....|....|....|....|....|....|....|....|....|....|....|....|
NP_173321.4 ICS2[Arabidopsis t.] CGCPVEEARLLIKQIESFDRGMYAGPIGFFGGGESEFSVGIRSALVEKGLGALIYAGTGI
A0A6N2L7G9 ICS [Salix v.]         ..F.T.......TES.V.........V.W........A.......................
XP_009104748.1 ICS1 [Brassica r. ..L.A.........E............V.....E....A.......................
XP_006302076.1 ICS1[Capsella r.] ..L.A.........E............V.....E....A.......................
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Figure 2a. Amino Acid (AA) diversity of plant ICS enzymes. AAs of (1-21) are indicated in color block letters; dots indicate the same AA aligned to the sequence in the first row. Protein blast (*Blast*p) was run by NCBI server, and the alignments were computed by *BioEdit* computer program (Hall, 1999) and aligned to ICS2 of *Arabidopsis thaliana* (Rédei, 1975). Protein sequences of related species of *Salix viminalis* (UniProtKB), *Brassica rapa* (NCBI), and *Capsella rubella* (NCBI) (stretches from 421 to 540 AAs) are compared, and genbank accession numbers are indicated.

Plant chloroplast genome (*i.e.*, plastome) of higher plants, *e.g.*, of *Arabidopsis* (Rédei, 1975) carries two ICS genes of *At*ICS1 (*At*1g74710; gene length 2.021 nt; protein EC 5.4.4.2; NP_565090; protein length 569 AA); and *At*ICS2 (*At*1g18870; gene length 1,689 nt; protein EC 5.4.4.2; NP_173321.4; protein length 562 AA) (Fig. 2a, Table 1) (Chen *et al.*, 2009; Macaulay *et al.*, 2017).

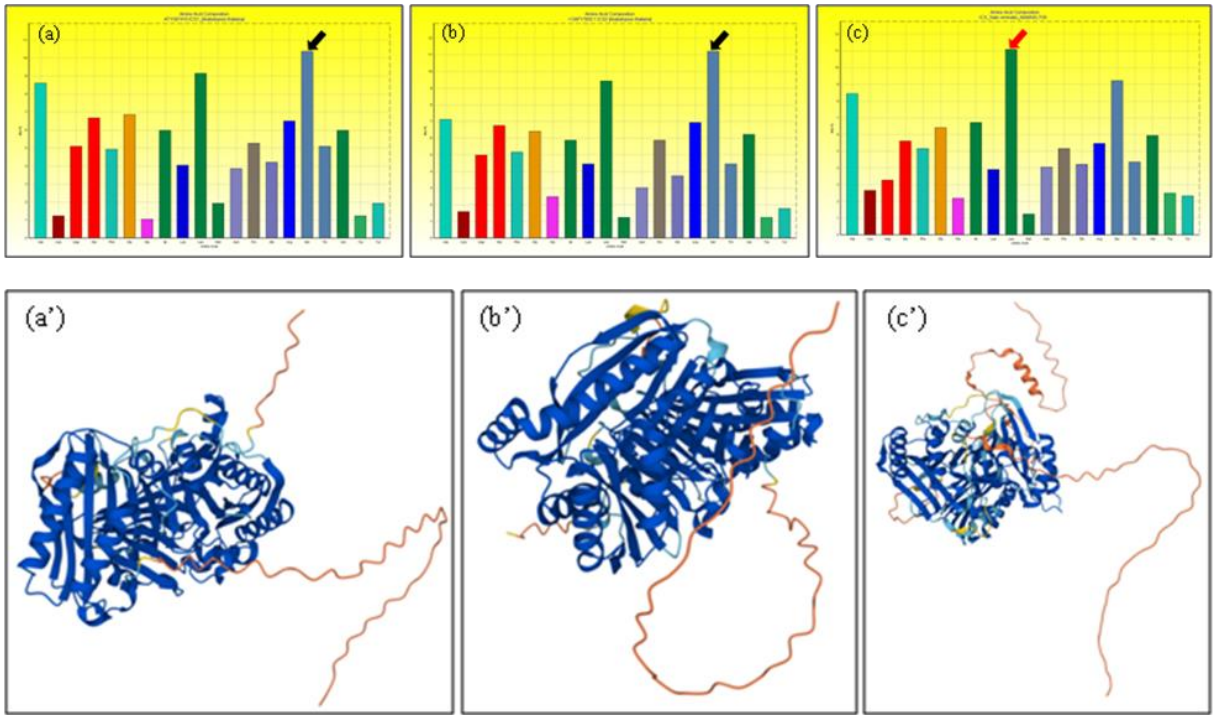

Figure 2b. Comparisons of amino acid compositions, *upper panels*, [*(a), (b), (c)*], and the 3D enzyme structures, *lower panels*, [*(a'), (b'), (c')*] of the enzyme *At*ICS1 *(a)* (gene length 2,021 nt; EC 5.4.4.2; enzyme length 569 AA) and *At*ISC2 *(b)* (gene length 1,689 nt; EC 5.4.4.2; enzyme length 562 AA) of *Arabidopsis thaliana* (Rédei, 1975); and compared to ICS of *Salix viminalis* (*SVim*_LOCUS128894; enzyme A0A6N2L7G9; enzyme length 640 AA) [*(c)* and *(c')*] (UniProtKB). Protein sequences were downloaded from NCBI and UniProtKB servers, and the figures were edited by the BioEdit computer program, *up*, (Hall, 1999). The 3D structure databases, *down*, of Q9M9V6 *(a')*, Q9S7H8 *(b')* and A0A6N2L7G9 *(c')* were downloaded from UniProtKB server. The 3-, and 1-letter codes of AAs are: *Alanine (Ala, A). Arginine (Arg, R). Asparagine (Asn, N). Aspartate (Asp, D). Cysteine (Cys, C). Glutamine (Gln, Q). Glutamate (Glu, E). Glycine (Gly, G). Histidine (His, H). Isoleucine (Ile. I). Leucine (Leu, L). Lysine (Lys, K). Methionine (Met, M). Phenylalanine (Phe, F). Proline (Pro, P). Serine (Ser, S). Threonine (Thr, T). Tryptophan (Trp, W). Tyrosine (Tyr, Y). Valine (Val, V).*

Sequence similarity analyses revealed that *Atics*1 gene has 13 coding regions (*i.e.*, exons) compared to *Atics*2, which has 15 exons (Garcion *et al.*, 2008). Several plant genomes sequenced (*e.g., Populus, Oryza, Ricinus,* and *Vitis*) showed a single copy ICS gene (Yuan *et al.*, 2009) also encoded in the chloroplast genome. However, it should be noted that a chloroplast (cp) encoded gene in a plant cell is present in at least 2500 copies, which comes from a general equation of 50 cpDNA in a cp x 50 cp in a cell.

Amino acid sequence alignments (Gyulai *et al.*, 2018) of plant ICS enzymes (*Syn., Salicylate_synthase*; InterPro *IPR019996*) analyzed here showed different ranges of amino acid diversity (Fig. 2a, 2b). However, dendrogram analysis (Fig. 2c) significantly discriminated the two plant ICS1 and -2 enzyme families.

The comparison of the amino acid composition of both *At*ICS1 and *At*ICS2 enzymes showed high levels of *serine* contents as 10.37 Mol% (*At*ICS1) and 11.21 Mol% (*At*ICS2) (Fig. 2b / a and b). Nevertheless, in *Salix viminalis* (A0A6N2L7G9, UniProtKB, 640 AA) the leucine content showed the highest level (11.09 Mol%) (Fig. 2b / c). The images of 3D enzyme structures (Fig. 2b / a', b', and c') showed distinct differences among the three ICS enzymes studied.

BLOCKING THE BIOSYNTHESIS OF SA IN PLANTS

Glyphosate, a broad-spectrum, nonselective, post-emergence herbicide (isolated/synthesized first from soil bacteria by Franz, J. E., 1970, and manufactured later by Monsanto Co. USA, from 1973 as *RoundUp* herbicide) was shown to block both routes of SA biosynthesis (Fig. 1b) by inhibiting the synthesis of *chorismic acid* from *shikimic acid* at the key enzyme EPSPS (*5-EnolPyruvylShikimate-3-Phosphate Synthase*) (UniProtKB P05466·AROA_*AraTh*, 520 AA, Mass 55.734 Da, EC 2.5.1.19, TAIR *At*1g48860) (Fig. 3).

The name *shikimate* comes from the Japanese name 'shikimi' of *Illicium anisatum* (*Eng*., Japanese star anise; *Hung*., csillagánizs). The name *chorismate* originates from the old Greek word 'to separate' as *chorismate* is at the branch point to the three aromatic amino acids biosynthesis (*Phe*, *Trp*, and *Tyr*). The final consequence of weed killing effect of glyphosate is that the weeds die of the lack of aromatic amino acids in proteins (WHO, 1994).

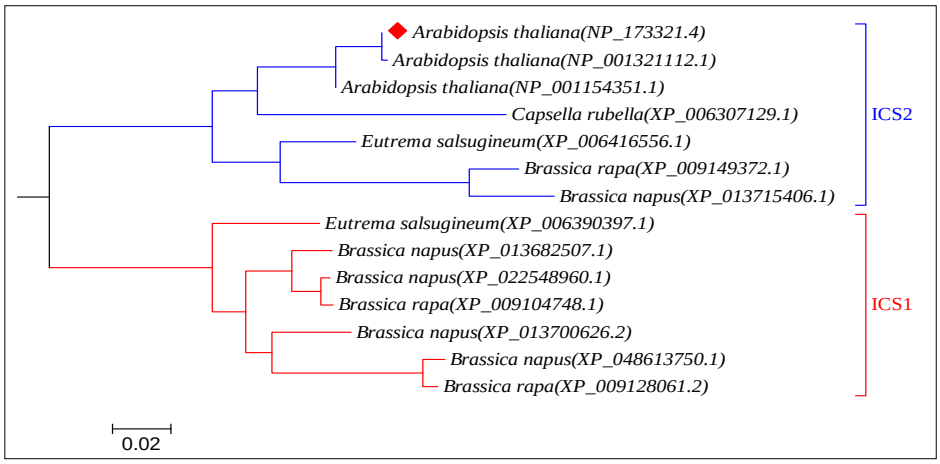

Figure 2c. Protein sequence (AA) similarity dendrogram (edited by NJ_Neighbor Joining algorithm; Distance_Kimura-protein; by NCBI server) of plant ICS1 and ICS2 (*Isochorismate synthase*1 and -2) enzymes. Protein sequences were downloaded from NCBI and aligned to that of *At*ICS2 AA sequence of *A. thaliana* (Rédei, 1975) (labeled by ♦). The distance tree was finally edited by MEGA7 (Kumar *et al.*, 2016) computer program. Plant names and genbank accession numbers are indicated.

A new concern of EPSPS was detected in glyphosate-resistant (GR) weeds which survive the glyphosate treatment by increased numbers of *EPSPS* gene copies and, consequently, increased amount of EPSPS enzyme, with a final glyphosate resistance. Tandem *EPSPS* gene duplications/amplifications were found to be generated by transposons (Alzohairy *et al*., 2014). *E.g.*, in GR *Kochia scoparia* weed (*Hung.*, seprőfű), the number of *EPSP* genes increased to 3 to 11 times higher compared to glyphosate-sensitive plants. GR *Amaranthus palmeri* weed of "giant weeds" showed hundreds of multiplied *EPSPS* gene. By these resistant weeds, a new type of *convergent evolution* was identified without any mutations in the *EPSP* gene itself (*In:* Patterson *et al*., 2018).

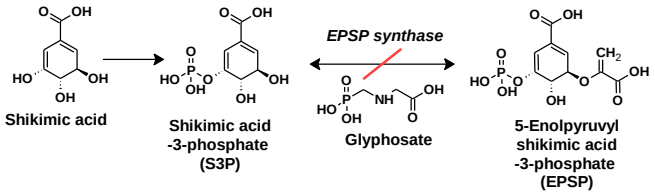

Figure 3. The phytotoxic site of the herbicide glyphosate (Monsanto Co., USA, 1973) by inhibiting the enzyme EPSP synthase (EC 2.5.1.19; TAIR *At*1g48860) (after Kőmíves and Schröder, 2016).

METABOLISM OF SA IN PLANTS

Glycosides. Plant cells can convert SA to storage metabolites, such as *salicin* (*SA-β-glucoside*) (Fig. 1a and 4) and *MeSAG* (*methyl salicylate 2-O-b-D-glucoside*) (Fig. 4). In *Arabidopsis*, two homologous enzymes UGT74F1 (UDP-glycosyltransferase 74 F1 of *A. thaliana*; NP_181912.1; 449 AA), and UGT74F2 of *A. thaliana* (OAP07463.1; 449 AA) transfer glucose from UDP-glucose to SA to create SA conjugates. These enzymes also catalyze the synthesis of SA glucosides (SAG) and SA glucose ester (SGE) (George Thompson *et al.*, 2017) (Fig. 4).

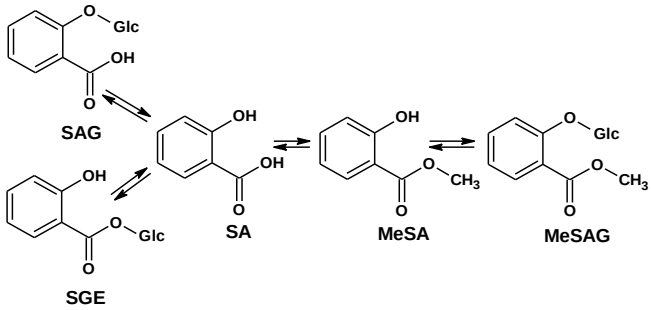

Figure 4. Central pathways of salicylic acid (*SA*) metabolism in plants (*SAG*: SA-2-O-b-D-glucoside. *SGE:* SA glucose ester. *MeSA:* methyl salicylate. *MeSAG:* methyl salicylate 2-O-b-D-glucoside.) (after Rivas-San Vicente and Plasencia, 2011).

ROLE OF SA IN PLANT DISEASE RESISTANCE

In plants, the endogenous and exogenously applied SA has been reported with numerous beneficial effects (White, 1979; Janda *et al.*, 1999), including the participation in local and *systemic acquired resistance* (SAR) against viral, bacterial, and fungal pathogens and pests, as first was documented by Bernard (1911).

The term SAR was coined by Ross (1961; *In:* Király *et al.*, 1972; Egyedi *et al*., 1992), coupled with hypersensitivity (HR, Stakman, 1915; *In:* Kömíves and Király, 2019). Further roles of SA in plant seed germination, flowering, and senescence were summarised by Rivas-San Vicente and Plasencia (2011).

VOLATILE SA METABOLITES

The volatile *MeSA* and *MeSAG* (Park *et al*., 2007) (Fig. 4) are synthesized in a reaction catalyzed by the enzyme *Salicylate/benzoate carboxyl methyltransferase* (NCBI NP_187755. UniProtKB/Swiss-Prot Q6XMI3.1. 378 AA).

Nine different *MeSA*-glycosides have been isolated from plants, mainly from the species of *Gaultheria, Camellia, Polygala, Filipendula, Passiflora*, and grapes *Vitis riparia* and *Vitis vinifera*. Recently, six of these glycosides were determined in Italian white wines (*e.g., MeSA-rutinoside, MeSA-gentiobioside, MeSA-lactoside, etc.*) (Carlin *et al.*, 2019). The smell of *MeSAG* was found to be balsamic-sweet-aromatic with a wintergreen-mint-fresh green character (Mansfeld *et al.*, 2011).

ELICITOR EFFECT OF THE SA METABOLITES

MeSAG may act as a signal molecule within and between plants (Liu *et al.*, 2018). By this way, *MeSAG* could be an initiator of allelopathy (a term coined by Hungaro-Austrian Molisch (1938) as well as an elicitor of plant disease resistance (*In:* Kömíves and Király, 2019).

For elicitor-induced SA synthesis, two HypersenSitivity-Related genes *HSR*201 and *HSR*203J were found to be regulated under transcription activators of *Nt*CBP60g (CBP: *Calmodulin Binding Protein*, F4K2R6, 563 AA, *At*) and *Nt*SARD1 (SARD: *Systemic Acquired Resistance Deficient*1 protein, Q9C9T2, 451 AA, *At*) in *Nicotiana tabacum* and -*bentamiana* (Takagi *et al.*, 2022).

Gene	Enzyme	EC #	Reaction
*At*1g74710	ICS1	EC 5.4.4.2	Chorismate → Isochorismate
*At*1g18870	ICS2	EC 5.4.4.2	Chorismate → Isochorismate
*At*3g29200	CM1	EC 5.4.99.5	Chorismate → Prephenate
*At*1g10870	CM2	EC 5.4.99.5	Chorismate → Prephenate
*At*2g37040	PAL1	EC 4.3.1.24	Phenylalanine → trans-Cinnamic acid
*At*3g53260	PAL2	EC 4.3.1.24	Phenylalanine → trans-Cinnamic acid
*At*5g04230	PAL3	EC 4.3.1.24	Phenylalanine → trans-Cinnamic acid
*At*3g10340	PAL4	EC 4.3.1.24	Phenylalanine → trans-Cinnamic acid
*At*AIM1			trans-Cinnamic acid → BA
*At*2g43840	UGT74F1	EC 2.4.1.237	SA → SAG
*At*2g43820	UGT74F2	EC 2.4.1	SA → SGE
At4g23570	SGT1A		SA → SAG
*At*3g11480	BSMT1	EC 2.1.1.273	SA → MeSA, BA → MeBA
*At*2g23620	MES1	EC 3.1.1	MeSA → SA
*At*2g23600	MES2	EC 3.1.1	MeSA→ SA
*At*2g23580	MES4	EC 3.1.1	MeSA → SA
*At*2g23560	MES7	EC 3.1.1	MeSA → SA
*At*4g37150	MES9	EC 3.1.1	MeSA → SA
*At*4g27260	GH3.5 / WES1	EC 6.3.2	SA → SA-Asp
*At*2g03760	SOT12	EC 2.8.2	SA → SA-503
			BA → SA (BA 2-hydroxylase)
PBS3			Isochorismate → Isochorismate-9-glutamate
EPS1			Isochorismate-9-glutamate → SA
	NPR1 NPR3/NPR4		SA receptor(s)
EDS5			Isochorismate transporter from chlp to cytosol

Table 1. Genes of enzymes participating in salicylate (SA) biosynthesis and metabolism in *Arabidopsis thaliana* (*At*) (Rédei, 1975). CM: Chorismate mutase. ICS: *Isochorismate synthase*. PBS3: *Substituted benzoates-glutamate ligase*. EPS1: *Enhanced Pseudomonas Susceptibility*1 enzyme. PAL: *Phenylalanine ammonia-lyase*: UGT74F1: *UDP-glycosyltransferase* 74 F1. UGT74F2: *UDP-glycosyltransferase* 74 F2. SGT1A: *phosphatase-like* protein. MES1-MES9: *carboxyesterase* enzyme.

CONCLUSIONS

Genetics, physiological roles, biosynthesis, and metabolism of salicylic acid (SA) and its metabolites in plants still show several unrevealed questions. Our results presented here aimed to find answers to some of them at gene and enzyme levels.

One of the double biosynthetic routes of SA synthesis is catalyzed by ICS (*Isochorismate synthase*) enzymes (ICS1 and -2), which were found to have diverse amino acid (AA) compositions; however, the protein sequence similarity dendrogram, and protein 3D models of the ICS enzymes showed significant differences.

The new achievements in the study of the roles of volatile SA metabolism and the roles of EPSPS enzyme, including the significance of the new glyphosate-resistant "*giant weeds*" developed by *EPSPS* gene amplifications, have also been indicated.

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