Content, Composition, and Biosynthesis of Anthocyanin in Fragaria Species: A Review

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Abstract. Anthocyanins are responsible for fruit coloration and are beneficial to human health. The fruits of wild strawberry (Fragaria ×ananassa) varieties are colorful, a trait that attracts consumers. The fruits of wild Fragaria species, close relatives of the cultivated strawberry, vary in color. In this review, we describe the content and composition of anthocyanins in cultivated and wild strawberry varieties. We also explore the biosynthetic pathway of anthocyanins, including their transcriptional regulation mechanisms. Additionally, we discuss the effect of environmental factors on anthocyanin accumulation. This review will inform further studies toward developing anthocyanin-rich strawberries via environmental control and exogenous application of compounds.

Anthocyanins are one of the pigments contributing to fruit color (Li et al. 2019a). In plants, anthocyanins attract pollinators and seed dispersers and enhance plant tolerance to biotic and abiotic stress (Krüger et al. 2021; Treutter 2005). Moreover, anthocyanins are flavonoids with strong antioxidant and free radical scavenging properties (Garcia and Blessos 2021; Kong et al. 2003). Fruit color is a crucial attribute influencing consumer preferences (Li et al. 2018). Anthocyanins also possess beneficial health effects against various disorders (Belwal et al. 2017; Hartman et al. 2006; Henriques et al. 2020; Jayaprakash et al. 2005, 2006; Seeram et al. 2006; Li et al. 2021a, 2021b; Mattioli et al. 2020; Shishrta et al. 2020). Therefore, anthocyanins have received significant research focus in recent years (Li et al. 2019b).

Cultivated strawberries (Fragaria ×ananassa Duch.), belonging to the genus Fragaria, are famous for their colorful appearance and delectable flavor. Currently, strawberries are grown worldwide, second only to grapes in global production (Dzhianfesova et al. 2020; Pillet et al. 2015; Qin et al. 2008; Sirijan et al. 2020; Zhao et al. 2021). Anthocyanin accumulation is responsible for the colorful appearance of strawberries (Sirijan et al. 2020; Zhao et al. 2021). Thus, various varieties of strawberries differ in appearance ranging from white to red, depending on the anthocyanin content and composition in their receptacles and achenes (Cheel et al. 2005; Lin et al. 2018; Wang et al. 2014). The wild Fragaria species, a close relative of cultivated strawberry, exhibits diverse appearances attributed to variations in anthocyanin accumulation in its fruits. For example, F. nitiderrinis produces white fruits (Shen et al. 2020; Zhang et al. 2020a), whereas F. penicilliferaa exhibits two morphs, red and white fruits (Duan et al. 2017, 2021). Therefore, there is a need for comprehensive exploration of the content, composition, and regulation of anthocyanin biosynthesis in Fragaria species. This will facilitate the development of anthocyanin-rich strawberries to meet the increasing demand for health-promoting compounds in the human diet (Xu et al. 2018).

Here, we describe the content and composition of anthocyanins in cultivated and wild strawberry species. Furthermore, we discuss the biosynthetic pathway of anthocyanins and their transcriptional regulation mechanisms. Finally, we highlight the effects of environmental factors, including abiotic stresses, on anthocyanin accumulation. This review summarizes vital information needed to develop anthocyanin-rich strawberries via environmental manipulations.

Anthocyanin Biosynthesis

Anthocyanin is synthesized via the phenylpropane pathway, and phenylalanine is the initial precursor in anthocyanin synthesis. Phenylalanine is catalyzed by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL) (Ariza et al. 2016). Subsequently, it enters the anthocyanin biosynthetic pathway, an extension of the general flavonoid pathway. Anthocyanin biosynthesis starts with the chalcone synthase (CHS)-mediated synthesis of naringenin chalcone from 4-coumaroyl-CoA and malonyl-CoA. Chalcone isomerase (CHI) then isomerizes naringenin chalcone to naringenin. Flavonone 3-hydroxylase (F3H) converts naringenin into dihydrokaempferol that is further hydroxylated by flavonoid 3′-hydroxylase (F3′H) or flavonol 3′,5′-hydroxylase (F3′5′H) into two other dihydroflavonols: dihydroquercetin and dihydromyricetin. The three dihydroflavonols are then converted by dihydroflavonol 4-reductase (DFR) into colorless leucoanthocyanidins, which are subsequently converted to colored anthocyanidins by anthocyanidin synthase (ANS) (Liu et al. 2018). Ultimately, colored anthocyanidins are glycosyltransfered by various flavonoid 3-O-glucosyltransferase (UGFT), whereas some are further acylated with aromatic acyl groups by acyltransferases (Liu et al. 2018).

Fruits contain six main anthocyanin pigments: cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (Chen et al. 2017). Cyanidin, pelargonidin, and delphinidin are synthesized from phenylalanine by various enzymes, and peonidin is synthesized from enzyme-modified cyanidin. Malvidin is synthesized from delphinidin (Fig. 1) (Shen et al. 2020). Anthocyanins are biosynthesized differently in various Fragaria species and

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varieties (Kim et al. 2015; Salvatierra et al. 2014; Shen et al. 2020; Simirgiotis et al. 2009). The most commonly occurring anthocyanins in strawberries are cyanidin and pelargonidin derivatives (Salvatierra et al. 2014). Cyanidin, pelargonidin, and delphinidin were identified in the red fruits of *F. pentaphylla*, whereas only cyanidin and delphinidin were detected in the white fruits of *F. nilgerrensis* (Shen et al. 2020). Additionally, rosinidin synthesized from enzyme-modified cyanidin was detected in *F. pentaphylla* and *F. nilgerrensis*, whereas petunidin was not detected (Fig. 1) (Shen et al. 2020).

**Anthocyanin Content and Composition**

**Anthocyanin content and composition in cultivated strawberries.** The content of anthocyanins varies among different varieties of cultivated strawberries (*F. ×ananassa*) (Buendia et al. 2010; Meyers et al. 2003; Skupień and Oszmianśki 2004). More than 25 anthocyanins have been detected in various strawberry cultivars via a high-performance liquid chromatography–diode array coupled to mass spectrometric detection (Silva et al. 2007). Notably, pelargonidin-3-glucoside, pelargonidin 3-O-rutinoside, and cyanidin-3-glucoside are the three main anthocyanins in strawberries (Dzhanfezova et al. 2020). Silva et al. (2007) studied the anthocyanin content of five strawberry cultivars (Camarosa, Carisma, Eris, Oso Grande, and Tudnew) and found that pelargonidin-3-glucoside accounted for 77% to 90% of the total anthocyanins, followed by pelargonidin-3-rutinoside (6% to 11%), and cyanidin-3-glucoside (3% to 10%). Dzhanfezova et al. (2020) evaluated anthocyanin levels in 12 noncommercial strawberry cultivars and found that pelargonidin-3-glucoside was the most abundant, accounting for 50% to 90% of the total anthocyanin content, followed by cyanidin-3-glucoside, which accounted for 1% to 47%, depending on the cultivar. Cyanidin-3-glucoside content has reduced significantly in modern cultivars (Kelebek and Selli 2011; Lin et al. 2018; Tonutare 2015). In addition, pelargonidin-3-rutinoside and pelargonidin-3-malonylglucoside accounted for 12.5% of the total anthocyanins, while pelargonidin-3-rutinoside only accounted for 3.1% in *F. ×ananassa* cv. Senga Sengana (Kjersti et al. 2005). Despite several reports on anthocyanin biosynthesis in strawberries, the studies were limited to fewer than 20 accessions. Transcriptome analysis of red and white strawberry cultivars revealed 70 differentially expressed genes involved in the anthocyanin pathway, which could potentially explain the white coloration of strawberry fruits (Zhao et al. 2021). Thus, additional studies are needed to identify the key genes responsible for anthocyanin accumulation and color formation in strawberry fruits.

**Anthocyanin content and composition in wild strawberries.** Anthocyanin levels and composition vary remarkably among wild and cultivated strawberries. Cyanidin-3-glucopyranoside is the most abundant anthocyanin in *F. chiloensis* (Cheel et al. 2005). Sondheimer and Karash (1956) and Tonutare (2015) reported that the major anthocyanins in...
The late-regulated biosynthesis genes (LBGs) of pelargonidin 3-glucoside and cyanidin 3-glucoside was 1.88 in *F. chiloensis* ssp. *chiloensis* f. *patagonica*, but 0.68 in *F. chiloensis* ssp. *chiloensis* f. *chiloensis*. Furthermore, Kim et al. (2015) examined the contents of pelargonidin 3-glucoside, pelargonidin-3-rutinoside, and cyanidin 3-glucoside of 14 *F. orientalis* varieties and concluded that the varieties had varying anthocyanin composition. Shen et al. (2020) detected 26 anthocyanins in *F. patagonica* and found that the content ratio of cyanidin, produced at a ratio of around 1:1. Similar to *F. patagonica*, *F. pentaphylla* and *F. chiloensis* ssp. *chiloensis* contained 26 anthocyanins in their composition. Shen et al. (2020) detected 27 single nucleotide polymorphisms within the FpDFR gDNA sequences between red and white fruits of *F. pentaphylla*, contributing to anthocyanin accumulation. The substrate specificity of FDFR is linked to the variations in anthocyanin composition in different Fragaria species (Miosic et al. 2014). Two DFR genes (DFR1 and DFR2) were identified in *F. ×ananassa* and *F. vesca*. Notably, DFR1 cannot catalyze dihydrokaemferol (the substrate for pelargonidin synthesis), whereas DFR2 exhibits a high affinity for dihydrokaemferol. Variations in the ratios of the two DFRs lead to different cyanidin to pelargonidin ratios between *F. ×ananassa* and *F. vesca* (Miosic et al. 2014). For example, the upregulation of DFR1 and silencing of F3H results in the accumulation of pelargonidin-based pigments in *F. ×ananassa*, whereas the enhanced expression of DFR2 and F3H promotes the synthesis of cyanidin-based pigments in *F. vesca* (Miosic et al. 2014).

EBGs appear to regulate anthocyanin biosynthesis in strawberries indirectly. Specifically, the silencing of CHS or F3H genes has been shown to decrease the anthocyanin content of strawberry fruits significantly (Hoffmann et al. 2006; Jiang et al. 2013). F3H and F3′5H catalyze the synthesis of cyanidin-based and delphinidin-based anthocyanins, respectively (Sei et al. 2007). These enzymes facilitate the hydroxylation of the anthocyanin B-ring, synthesized from 4-coumaroyl-CoA produced from phenylalanine via the shikimate pathway (Nabavi et al. 2020). Increasing hydroxylation of the B-ring affects the hue of the pigment and causes a shift from the red end of the visible spectrum to the blue (Schwill et al. 2014; Tanaka 2006). F3H expression varies significantly between different Fragaria species/gentotypes during fruit development, impacting the composition of the two major anthocyanins (cyanidin and pelargonidin derivatives) (Thill et al. 2013).

Glutathione S-transferase (GST) is an enzyme involved in cellular detoxification processes and has been implicated in plant stress responses (Allocati et al. 2018). Lin et al. (2020) found that FaGST1, FaGST37, FaGST39, FaGST73, and FaGST79 may indirectly promote vacuolar anthocyanin accumulation in cultivated strawberries. Moreover, the expressions of these five FaGST genes were significantly correlated with the expressions of other anthocyanin biosynthetic structural genes (e.g., FaCHL, FaCHS, and FaANS), indicating their involvement in anthocyanin accumulation in strawberries.
of ANS and downregulation of ANR and LAR, leading to increased anthocyanin content (Salvatierra et al. 2014). However, FaMYB1 did not repress anthocyanin synthesis in F. vesca (Roy et al. 2018) as it does in F. ×ananassa (Aharoni et al. 2001; Hu et al. 2018) and F. chiloensis (Salvatierra et al. 2014). Researchers have revealed that the regulatory mechanism of MYB1 on anthocyanin biosynthesis varies between F. ×ananassa and F. chiloensis. However, silencing of the MYB1 gene suppresses the expression of LAR, an enzyme that catalyzes proanthocyanidin synthesis, in both Fragaria species (Salvatierra et al. 2014). These results indicate that MYB1 negatively controls anthocyanin biosynthesis in strawberry fruits by regulating the expression of anthocyanin/proanthocyanidin biosynthetic structural genes. This further affects the synthesis direction of the intermediate metabolites (Salvatierra et al. 2014).

Moreover, genome-wide analysis of the MYB gene family in the octoploid strawberry revealed that FAFMYB28, FAFMYB54, and FAMYB576 potentially regulate anthocyanin biosynthesis (Liu et al. 2021). Meanwhile, FAFMYB5, FAFMYB11, and FAFMYB9 were demonstrated to repress anthocyanin biosynthesis in the octoploid strawberry (Hossain et al. 2018; Schaar et al. 2013).

The role of basic helix-loop-helix transcription factors in anthocyanin biosynthesis. The basic helix-loop-helix (bHLH) transcription factor family is the second-largest transcription factor family in plants. It is characterized by the conserved bHLH domain and plays a central regulatory role in many plant biological processes (Zhao et al. 2018). Four bHLHs (FabHHLH23, FabHHLH29, FabHHLH80, and FabHHLH88) have been predicted to regulate anthocyanin biosynthesis in strawberries (Zhao et al. 2018). Furthermore, Li et al. (2020) found that FabHHLH9 is a positive regulator of anthocyanin biosynthesis in F. vesca. However, FabHHLH9 can only promote the transcription of FaMYB10 to activate FaDFR expression in the presence of WDG (Li et al. 2020). This is consistent with the previous reports that the genes coding for the key enzymes in the anthocyanin biosynthesis are mainly regulated at the transcriptional level by the MYB-bHLH-WD40 (MBW) complex (An et al. 2012; Espley et al. 2007; Yao et al. 2017; Zhou et al. 2019).

H15 belongs to the basic leucine zipper (bZIP) transcription factor and is light-inducible (Li et al. 2020). Under light conditions, H15 and MYB aggregate and directly bind the promoters of anthocyanin biosynthesis genes to promote anthocyanin synthesis (Takos et al. 2006; Stracke et al. 2010). Li et al. (2020) further confirmed that FaH15 and FaHHLH9 specifically bind the promoter region of some key enzyme genes, including FaDFR, to activate their expression. For example, FaDFR expression is activated through the heterodimer formed between FaH15 and FabHHLH9. Wang et al. (2014) also found that FabHHLH3, a potential partner of FabMYB10, can regulate anthocyanin structural genes, resulting in anthocyanin accumulation. Besides, FabHHLH3 and FabHHLH3-delta are potential positive regulators, whereas FabHHLH3 is a potential negative regulator of anthocyanin biosynthesis in the high-anthocyanin strawberry cultivars (Hossain et al. 2018).

The role of other transcription factors in anthocyanin synthesis. More recently, many other families of transcription factors have been demonstrated to modulate anthocyanin (An et al. 2017; Duan et al. 2017). The RAV group (related to ABI3/VP1) of transcription factors (TFs) plays multifaceted roles in plant growth, development, and responses to environmental stresses. Zhang et al. (2020b) found that FaRAV1 had the highest transcriptional activation effect on the promoter of FaMYB10, a key activator of anthocyanin biosynthesis. The study also reported that silencing FaRAV1 through transient RNA interference decreased FaMYB10 expression and anthocyanin content in F. ×ananassa fruits. Additionally, transcriptional analysis of FaRAV1-overexpressing strawberry fruit revealed that transcripts of phenylpropanoid and flavonoid biosynthetic pathway genes were upregulated. FaRAV1 stimulates anthocyanin accumulation in strawberries by either directly activating anthocyanin pathway gene promoters or upregulating FaMYB10 (Zhang et al. 2020b).

The plant-specific transcription factor Teosinte branched1/Cycloidea/Proliferating cell factors (TCP) is crucial in plant growth and development. FaTCP9 participates in the biosynthesis of abscisic acid (ABA) and anthocyanins to regulate fruit ripening. Transcription analysis showed that FaTCP9 could affect the expression of ABA signaling-related genes (FaNCED1, FaPYR1, FaSnRK2, and FaAB5). A yeast two-hybrid assay revealed that FaTCP9 interacts physically with FaMYC1 to modulate anthocyanin biosynthesis, indicating that FaTCP9 promotes fruit ripening by regulating the biosynthesis of ABA and anthocyanins (Xie et al. 2020). Notably, FaWD40-1 is a potential negative regulator of anthocyanin biosynthesis in high-anthocyanin strawberry cultivars (Hossain et al. 2018).

Factors Affecting Anthocyanin Biosynthesis in Fragaria Species

Light. Light intensity and quality, including shading (Antonnen et al. 2006), ultraviolet-B radiation (Josutti et al. 2010; Ordidge et al. 2010; Tsompatsidis et al. 2011), and blue and red LED light (Zhang et al. 2018a, 2018b, 2018c), significantly affect anthocyanin biosynthesis (Cominelli et al. 2008; He and Giusti 2010). Anthocyanins protect plants from ultraviolet damage (Zoratti et al. 2014). Light treatment (blue, green, and red light), especially blue light, can increase anthocyanin synthesis in the post-harvest strawberry fruits during storage by inducing the activities of CHS, F3H, DFR, ANS, and UFGT (Kadomura-Ishikawa et al. 2013). Blue light-irradiated (40 μmol·m⁻²·s⁻¹ blue light irradiation) fruits of F. ×ananassa cv. Fengguang had higher anthocyanin content accompanied by increased activities of TAL, PAL, C4H, and 4CL (Xu et al. 2018). Light treatment also increased the expression of MYB10 in F. ×ananassa and F. vesca, thereby increasing the expression of anthocyanin structural genes and anthocyanin biosynthesis (Kadomura-Ishikawa et al. 2015a; Xu et al. 2018). Recent studies have shown that photoreceptors, such as Phototropin 2 (PHOT2), are crucial in mediating anthocyanin accumulation in response to light (Sharma et al. 2021; Zhang et al. 2016). Overexpression of PHOT2 increases anthocyanin content in its leaves and fruits in F. ×ananassa (Sharma et al. 2021).

Temperature. The synthesis of anthocyanin is also affected by temperature. When exposed to cold temperatures, plants increase anthocyanin biosynthesis to improve their survival under cold stress (Catala et al. 2011). Conversely, suppressing anthocyanin biosynthesis might enhance survival under high-temperature stress (Kim et al. 2017). In addition, Kim et al. (2017) discovered that high temperature induces the degradation of HYS protein through COP1, enhancing the expression of the negative regulator and reducing the expression of anthocyanin biosynthetic genes.

Studies have shown that temperature affects anthocyanin accumulation in strawberry fruits. A positive correlation between anthocyanin contents and the temperature has been observed in strawberries grown in controlled environments (Balasooriya et al. 2020; Josutti et al. 2011; Wang and Zheng 2011) and under natural conditions (Cervantes et al. 2020; Josutti et al. 2012). Wang and Zheng (2011) found that increasing the night temperature from 12 to 22 °C, with the day temperature kept constant at 25 °C, significantly increased anthocyanin content in F. ×ananassa. Balasooriya et al. (2020) also found that a day temperature of 30°C could significantly increase anthocyanin content in strawberry fruits than 25°C. Low-temperature stress (4°C) increased the expression of anthocyanin structural genes and anthocyanin accumulation in F. ×ananassa fruits by regulating the expression of FaMYB10 and FaMYB1 (Zhang et al. 2018c). Furthermore, low temperature induced anthocyanin production
in strawberries, alleviating oxidative damage (Zhang et al. 2018c). The increase in anthocyanin content at low temperatures can be attributed to the modulation of ANS and UFGT structural genes by the regulatory genes (MYB10 and MYB11) (Zhang et al. 2018c).

Temperature also affects anthocyanin composition. For example, fruits grown at higher temperatures exhibited increased levels of total anthocyanin and pelargonidin-3-glucoside, the most abundant anthocyanin in strawberries (Balasooriya et al. 2020). CO2. Different studies have reported inconsistent results regarding the effect of CO2 on anthocyanin biosynthesis in strawberries. Some studies have shown that elevated CO2 concentration can increase anthocyanin accumulation in F. × ananassa (Balasooriya et al. 2020; Choi and Kang 2018; Wang et al. 2003). Shin et al. observed higher levels of anthocyanins in air-stored strawberries than in CO2-stored strawberries (Shin et al. 2008). A high concentration of CO2 can increase the carbon supply in the growth environment, leading to higher carbon availability in plants. High carbon availability is associated with more carbohydrate accumulation and enhanced synthesis of carbon-based secondary products, especially soluble phenols, and condensed tannins (Balasooriya et al. 2020).

However, some studies have reported that high CO2 levels can negatively affect anthocyanin accumulation. Li et al. (2019a) found that elevated CO2 (20% CO2 treatment) lowers anthocyanin content and the activity of PAL, CHS, 4CL, and CHS. The elevated CO2 levels also suppressed the expression of 13 genes involved in the phenylpropanoid and flavonoid biosynthetic pathways, suggesting that high CO2 concentration may suppress anthocyanin biosynthesis by regulating the flavonoid pathway. In contrast, Blanch et al. (2012) found that anthocyanin accumulation was not reduced in strawberries treated with 20% CO2; however, flavonoid production was redirected toward catechin and procyanidin B3 accumulation in 40% CO2-treated fruits, with a sharp decrease in anthocyanin levels. The study also showed that catechin induction by high CO2-treated could protect strawberries from fungal decay.

In addition, the effect of CO2 on anthocyanin biosynthesis has been shown to vary among different varieties. For instance, Gil et al. (1997) reported that CO2 had a minimal effect on the anthocyanin contents of the external tissues but induced a remarkable decrease in anthocyanin accumulation in the internal tissues.

Water and salt. Plants growing in the field experience multiple stresses during their development, compounded by the dramatic changes in global climate. A recent study revealed that water stress could increase anthocyanin content in F. × ananassa fruits (Adak et al. 2017). Similar results were observed in strawberry plants subjected to drought stress (Terry et al. 2007) or salt stress (Keutgen and Pawelzik 2008). Mild drought and salt stresses have also been shown to increase the content of phenolics, anthocyanins, and L-ascorbic acid. Drought and salt stresses are also associated with increased anthocyanin content and antioxidant activity through an ABA-dependent mechanism in F. × ananassa fruits (Perin et al. 2019). Perin et al. (2019) found that drought or salt-stress-induced anthocyanin accumulation and increased levels of ABA and its derivatives (phasic and dehydrophasic acids), associated with the phenylpropanoid and flavonoid pathways, activate the expression of several anthocyanin synthesis genes.

**Exogenous compounds.** Exogenous compounds, such as sucrose (Li et al. 2019b) and ABA (Li et al. 2012; Jia et al. 2011), were found to induce anthocyanin synthesis. Li et al. (2019b) found that exogenous sucrose application on strawberry fruits during storage can increase the accumulation of four pelargonidin derivatives, including pelargonidin 3-glucoside, pelargonidin 3-rutinoside, pelargonidin 3-malonylglucoside, and pelargonidin 3-methylmalonylglicoside. Moreover, the accumulation of pelargonidin derivatives was involved in activating the pentose phosphate, shikimate, phenylpropanoid, and flavonoid pathways (Li et al. 2019b).

Furthermore, Cao et al. (2010) found that applying 0.2 g/L of benzo-thidiazol-7-carboxylic acid 5-methyl ester (BTH) on strawberry fruits for 10 days at 1 °C increases anthocyanin content. This can be attributed to the activation of BTH-related enzymes, including G6PDH, SKDH, TAL, PAL, C4H, and DFR (Cao et al. 2010).

**Conclusion**

Anthocyanin content and composition vary among cultivated and wild strawberry varieties. The three major anthocyanins in strawberries are pelargonidin-3-glucoside, pelargonidin 3-O-rutinoside, and cyanidin-3-glucoside. However, the biosynthetic pathways of anthocyanins in strawberries need further exploration. Anthocyanin biosynthesis in strawberries is regulated by structural genes, including EBGs and LBGs. Transcriptome analyses specifically those belonging to the R2R3-MYB, bHLH, and WD40 families, are crucial in anthocyanin biosynthesis regulation. However, more novel transcription factors involved in anthocyanin biosynthesis need to be identified and characterized (Lin et al. 2018). Genetic engineering and hybrid screening are increasingly being used to enhance anthocyanin content in strawberries for improved fruit quality (Qin et al. 2008; Siriwardana et al. 2020; Tang et al. 2018). When food becomes a medicine. Nutrients. 2017. Dietary anthocyanins and insulin resistance: a review. Nutrients. 9(10):1111. https://doi.org/10.3390/nu9101111.

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