Evolution and roles of cytokinin genes in angiosperms 1: Do ancient IPTs play housekeeping while non-ancient IPTs play regulatory roles?

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Abstract
Isopentenyltransferase (IPT) genes, including those encoding ATP/ADP-IPTs and tRNA-IPTs, control the rate-limiting steps of the biosynthesis of N⁶-(Δ²-isopentenyl)adenine (iP)-type and trans-zeatin (tZ)-type cytokinins and cis-zeatin (cZ)-type cytokinins, respectively. However, the evolution and roles of these IPTs in angiosperms are not well understood. Here, we report comprehensive analyses of the origins, evolution, expression patterns, and possible roles of ATP/ADP-IPTs and tRNA-IPTs in angiosperms. We found that Class I and II tRNA-IPTs likely coexisted in the last common ancestor of eukaryotes, while ATP/ADP-IPTs likely originated from a Class II tRNA-IPT before the divergence of angiosperms. tRNA-IPTs are conservatively retained as 2–3 copies, but ATP/ADP-IPTs exhibit considerable expansion and diversification.

Additionally, tRNA-IPTs are constitutively expressed throughout the plant, whereas the expression of ATP/ADP-IPTs is tissue-specific and rapidly downregulated by abiotic stresses. Furthermore, previous studies and our present study indicate that ATP/ADP-IPTs and their products, iP/iZs, may regulate responses to environmental stresses and organ development in angiosperms. We therefore hypothesize that tRNA-IPTs and the associated cZs play a housekeeping role, whereas ATP/ADP-IPTs and the associated iP/iZ-type cytokinins play regulatory roles in organ development and stress responses in angiosperms, which echoes the conclusions and hypothesis presented in the accompanying study by Wang, X. et al. Evolution and roles of cytokinin genes in angiosperms 2: Do ancient CKXs play housekeeping roles while non-ancient CKXs play regulatory roles? Hort Res https://doi.org/10.1038/s41438-020-0246-z.

Introduction
Cytokinins (CKs) are a class of plant hormones that play essential roles in many aspects of plant development, including the delay of leaf senescence⁴, root proliferation⁵,⁶,⁷, apical dominance⁴, shoot meristem function⁵, regulation of reproductive meristem activity⁸, fruit development⁹, and nutritional signaling¹⁰,¹¹. In addition, CKs play important and complex roles in environmental stress responses¹²,¹³. There are two major types of naturally occurring CKs, the cis-zeatin (cZ) type and the iP [N⁶-(iP)/trans-zeatin (tZ)] type¹⁴. In many plants, iP and tZs are the most prevalent CKs in most tissues and stages of the lifespan, while cZ-type CKs are present only in minor quantities¹⁵. Recent studies have demonstrated that cZs are the predominant CKs in some plants, such as rice and maize, or in certain developmental stages associated with limited growth¹⁶. The presence of iP-type and tZ-type CKs can vary greatly between tissues, developmental stages, and environmental conditions¹⁷–¹⁹.

Isopentenyltransferases (IPTs) catalyze the first and rate-limiting step of CK biosynthesis. IPTs can be classified into the adenylate (ATP/ADP-IPTs and AMP-IPTs) and tRNA types. The IPTs that use ATP, ADP, or AMP as their preferred substrates produce iP-type and tZ-type CKs, while the tRNA-type IPTs, which transfer isopentenyl groups to...
the $N^6$ atom of adenines in tRNAs, produce cZ-type CKs. In Arabidopsis, nine IPT genes have been identified (AtIPT1–9). Among them, AtIPT1 and AtIPT3–AtIPT8 encode ATP/ADP-IPTs, and the other two AtIPT genes, AtIPT2 and AtIPT9, encode tRNA-IPTs. Mutations in two tRNA-type IPT genes, AtIPT2 and AtIPT9, particularly in combination, result in plant chlorosis and a significant reduction in cZ-type CKs but do not affect the concentrations of iP$s$ and tZ$s$. By comparison, plants with mutant ATP/ADP-IPTs possess scarce amounts of iP-type and tZ-type CKs but contain slightly elevated levels of cZ$s$. These mutant plants exhibit increased lateral root primordia and density, fewer leaves, reduced inflorescence, and decreased number of vascular bundles and are unable to form cambium.

Genetically mutant plants with altered endogenous CK content display changes in plant tolerance to environmental stresses. A quadruple ATP/ADP-IPT Arabidopsis mutant atipt1;3;5;7 with deficient levels of iP$s$ and tZ$s$ but slightly increased levels of cZ$s$ displayed significantly enhanced salt and drought tolerance compared to that of wild-type plants. Plants overexpressing CK degradation genes (CK oxidase/dehydrogenase genes, CKX$s$), which contain substantially reduced levels of iP$s$/tZ$s$ and moderately reduced levels of cZ$s$, also show elevated tolerance to drought, salt, or heat stress. These studies show that iP and tZ CKs are negative regulators of plant adaptation to environmental stresses. However, it has also been reported that if a stress-associated or senescence-associated promoter is used to drive the expression of an AMP-IPT or ATP/ADP-IPT gene, improved plant tolerance to drought, heat, and other stresses is observed.

As previously reported, tRNA-IPT$s$ have been found in all major clades of bacteria and eukaryotes but not in archaea. In contrast, adenylate IPT$s$ show a fragmented distribution. The functionally confirmed ATP/ADP-IPT$s$ are found exclusively in flowering plants. Based on the tree topology and taxonomic composition of the IPT gene family, Lindner et al. have suggested that eukaryotic IPT$s$ most likely can be traced back to an ancestral α-proteobacterium that was involved in the initial endosymbiotic event that led to extant mitochondria. The ancestral eukaryotic IPT gene was duplicated, which resulted in two classes of tRNA-IPT$s$ (Class I and II). The Class II tRNA-IPT$s$ subsequently lost the capability to bind tRNAs and diversified into the extant ADP/ATP-IPT$s$ found in flowering plants. Recently, Nishii et al. performed a broader sampling of IPT genes and hypothesized that Class I tRNA-IPT$s$ represent the direct successors of bacterial miaA genes, while Class II tRNA-IPT$s$ are derived from eukaryotic genes.

In this study, we comprehensively analyzed the differences between ATP/ADP-IPT$s$ and tRNA-IPT$s$, including their taxonomic distribution, origin, evolutionary history, duplication mechanism, gene/protein structure, and expression patterns in tissues/organs and under various environmental stresses. Our results strongly suggest that tRNA-IPT$s$ in angiosperms can be traced back to the initial endosymbiotic event, while ATP/ADP-IPT$s$ are derived from tRNA-IPT$s$. In flowering plants, tRNA-IPT$s$ are conservatively retained and constitutively expressed in all tissues and under various environmental conditions. On the other hand, ATP/ADP-IPT$s$ have extensively expanded and functionally diverged, and their expression is tissue-/organ-specific and environmental stress-responsive. Our findings plus previously published results demonstrate that in angiosperms, tRNA-IPT genes and the associated cZ-type CKs most likely play housekeeping roles, while ATP/ADP-IPT genes and the associated iP/tZ-type CKs may be involved in regulating organ development and responses to environmental stresses.

**Results**

**Genome-wide identification of IPT genes among the three domains of life**

IPT genes have been identified in various groups of bacteria and eukaryotes but not in archaea. To verify the absence of IPT homologs in archaea, we used TBLASTN to identify the IPT domain in archaeal nucleotide databases. Putative IPT domains were found in two archaeal species, *miscellaneous Crenarchaeota group archaean SMTZ-80* and *candidate division MSBL1 archaean SCGC-AAA382N08*, indicating that IPT homologs are also present in archaea. The former archaeal species containing an IPT homolog belongs to the TACK superphylum, which has recently been proposed to be the origin of eukaryotes, and the latter is a member of the unclassified euryarchaeota.

We next performed a broad and balanced sampling of a total of 59 representative species from major lineages of all three domains of life (eukaryotes, bacteria, and archaea, Table S1). IPT homologs in these species were identified. We found that the number of IPT homologs varies considerably among the species in Plantae, ranging from one copy in *C. reinhardtii* (green algae) and *C. merolae* (red algae) to nine copies in *Arabidopsis thaliana* (eudicot) and 11 copies in *Z. maize* (monocot). By comparison, the number of IPT homologs in the other organisms, i.e., nonplant eukaryotes, bacteria, and archaea, does not vary greatly and ranges from 0 to 3 copies (Table S1). More than 90% (20/22) of the bacteria and nonplant eukaryotes sampled contain putative IPT gene(s) (mostly a single copy), while none of the IPT homologs could be found in the sampled archaea except the two identified by TBLASTN. Taken together, our results show that IPT homologs are distributed
sporadically in archaea but widely throughout bacteria and eukaryotes, and show significant expansions in land plants.

**Differences in the origin and distribution of tRNA-IPTs and ATP/ADP-IPTs**

To explore the evolutionary history of the IPT genes, an unrooted ML phylogeny comprised of IPT homologs from 59 representative species was constructed using PhyML (Fig. 1). Based on IPTs with known functions, the entire IPT gene family could be divided into three subfamilies, designated tRNA-IPT, ADT/ATP-IPT, and AMP-IPT. According to our phylogeny, the AMP-IPT subfamily includes only members of *D. discoideum* and several bacteria, including *Agrobacteria*, and the ATP/ADP-IPT subfamily is confined to seed plants (Table 1). In comparison, the tRNA-IPT subfamily has a much wider taxonomic distribution.

The two classes of tRNA-IPT genes (referred to as Class I and II hereafter) categorized by Lindner et al. are located in two clades and include members of various species (Fig. 1). The Class I tRNA-IPT homologs can be found in all supergroups of eukaryotes except Excavata (Table 1). In particular, every species containing plastids...
has at least one Class I tRNA-IPT (Table S1), revealing the universal distribution of this type of IPT gene in photosynthetic organisms. Within the phylogeny, all eukaryotic Class I tRNA-IPTs form a clade that branches with the tRNA-IPT homologs from bacterial species belonging to proteobacteria and four other phyla (Fig. 1 and Table S1). It is worth noting that these five bacterial phyla have relatively close relationships in the recent tree of life28. Therefore, the bacterial homologs of Class I tRNA-IPTs may be traced back to the ancestor of these proteobacteria-related bacterial species.

In contrast, the Class II tRNA-IPT subfamily includes homologs from seed plants and two prasinophyte algae in Plantae and all nonplant eukaryotic supergroups (Table 1). In particular, the Class II tRNA-IPTs and ATP/ADP-IPTs in seed plants form a clade that is a sister to that of the putative Class II tRNA-IPTs in prasinophyte algae and nonplant eukaryotes. This phylogenetic relationship indicates that these two types of IPT genes in seed plants have a common ancestor, and the ATP/ADP-IPTs are likely derived from the Class II tRNA-IPTs. Additionally, the eukaryotic Class II tRNA-IPT clade branches with a cluster of IPTs, including AMP-IPTs and bacterial and archaeal tRNA-IPTs. Hence, we categorized these bacterial and archaeal tRNA-IPTs as putative Class II members. The bacterial species containing these putative Class II tRNA-IPTs include cyanobacteria, actinobacteria, and other species except proteobacteria and those species that are phylogenetically closely related to proteobacteria.

In other words, all the bacteria sampled contain only one type of tRNA-IPT homolog, and those with the same type of tRNA-IPT homolog are phylogenetically more closely related than those without a homolog. In the sampled bacteria, there are four bacterial species with two IPT copies, all of which have one AMP-IPT homolog and one tRNA-IPT homolog of either of the two classes. In contrast, all the nonplant eukaryotes with more than one IPT copy contain both types of tRNA-IPTs, with only one exception. Moreover, these nonplant eukaryotic species belong to different supergroups that diverged at the base of the eukaryote phylogenetic tree, indicating that the coexistence of the two classes of tRNA-IPTs probably originated before the divergence of these supergroups.

### Differences in evolutionary patterns between tRNA-IPTs and ATP/ADP-IPTs in land plants

Our identification shows that the IPT genes have undergone considerable expansions, specifically in land plants. To further investigate the evolution of IPT genes in land plants, we identified 171 IPTs from 19 sequenced species belonging to every major lineage of land plants (Table S2) and constructed an unrooted ML tree using PhyML (Fig. 2a). According to the tree topology and that of the known IPTs, the land plant IPT gene family was classified into four groups: tRNA-IPT I, tRNA-IPT II, ATP/ADP-IPT I, and ATP/ADP-IPT II. The members of the tRNA-IPT I group are distributed throughout all the main lineages of land plants, while those of the tRNA-IPT

### Table 1 Phyletic distribution of ATP/ADP-IPT, AMP-IPT, and Class I and Class II tRNA-IPT homologs

| Domain   | Supergroup | Lineage          | ATP/ADP-IPT | tRNA-IPT | AMP-IPT |
|----------|------------|-----------------|-------------|----------|---------|
|          |            |                 | I           | II       |         |
| Eukaryota| Plantae    | Angiosperms     | +           | +        | +       |
|          |            | Gymnosperms     | +           | +        | -       |
|          |            | Lycophytes     | –           | +        | –       |
|          |            | Liverworts      | –           | +        | –       |
|          |            | Mosses          | –           | +        | –       |
|          |            | Green algae     | –           | +        | –       |
|          |            | Red algae       | –           | +        | –       |
|          | SAR        |                 | –           | +        | –       |
|          | Excavata   |                 | –           | –        | +       |
|          | Amoebozoa  |                 | –           | +        | –       |
|          | Opisthokonta|                | –           | +        | –       |
|          | Incertae sedis |           | –           | +        | –       |
| Bacteria |            |                 | –           | +        | +       |
| Archaea  |            |                 | –           | –        | +       |

Note: Plus (+) and minus (−) indicate whether the gene was detected in the corresponding lineage/supergroup/domain.
II group are confined to seed plants, which is consistent with the distribution of the two tRNA-IPT classes in the phylogeny shown in Fig. 1. The total number of tRNA-IPT genes in each flowering plant species is either two or three (Table 1), revealing that a conserved number of this type of IPT gene is retained in angiosperm genomes. However, six and five tRNA-IPT genes were found in Physcomitrella patens (moss) and Picea abies (gymnosperm), respectively, indicating that lineage-specific expansions have occurred in these two species/lineages.

The number of ATP/ADP-IPT genes varies from two copies in Amborella trichopoda to 21 copies in Medicago truncatula, which accounts for 40% of IPTs in the former and 91.3% of IPTs in the latter (Table 2). The high and increased percentages of ATP/ADP-IPTs indicate that the increased number of IPT genes in flowering plants is mainly due to the expansion of ATP/ADP-IPTs. According to the phylogeny, the two groups ATP/ADP-IPT I and II contain each of the two ATP/ADP-IPT genes in the basal angiosperm A. trichopoda (Fig. 2a), respectively, suggesting that two ancestral ATP/ADP-IPTs were likely present in the last common ancestor of angiosperms. In addition, the ATP/ADP-IPT I group can be further divided into four subgroups, Ia, Ib, Ic, and Id. The ATP/ADP-IPT Ia–Id subgroups contain only eudicot members, while the ATP/ADP-IPT Id subgroup contains exclusively monocots, reflecting the lineage-specific duplications of angiosperm ATP/ADP-IPTs (Fig. 2b and Table S3). Notably, PaeIPT3, the P. abies gene that is located in the ATP/ADP-IPT clade of the phylogeny shown in Fig. 1, branches with the tRNA-IPT II group in the land plant trees, which is supported by the low bootstrap value (Fig. 2a). Therefore, whether gymnosperms contain ATP/ADP-IPT homologs could not be determined from the phylogenetic analyses. Nevertheless, the existence and expansion of ATP/ADP-IPTs in flowering plants could be determined.

We further explored the duplication mechanisms responsible for the expansion of ATP/ADP-IPT genes in the 15 angiosperms using the MCScanX package. Each ATP/ADP-IPT gene was assigned to one of the five modes, including WGD/segmental duplication, tandem duplication, proximal duplication, dispersed duplication, and singleton (Table 2). Approximately 47.5% of the ATP/ADP-IPT genes from every dicot or monocot were shown to be involved in WGD gene duplications, demonstrating that WGD/segmental duplication is a primary mechanism. In contrast, tandem and proximal duplications accounted for 13.2% and 5.7% of the ATP/ADP-IPT duplications, respectively. In particular, 21 of the 23 tandem/proximal duplications, which belong to the ATP/ADP-IPT Ia subgroup, are derived from only two species, M. truncatula and S. lycopersicum. These results indicate that WGD/segmental duplication has extensively contributed to the expansion of ATP/ADP-IPT genes, while tandem duplication has given rise to lineage-specific expansions of the ATP/ADP-IPT genes in flowering plants.

**Differences in the gene and protein structures of tRNA-IPTs and ATP/ADP-IPTs**

We next compared the structural differences between tRNA-IPTs and ATP/ADP-IPTs, beginning with the
investigation of the exon–intron organization of IPT genes in 19 land plants (Fig. 2a). It is interesting to note that 77.7% of the 121 ATP/ADP-IPT genes contain no introns, and 85.2% of the remaining ATP/ADP-IPTs contain only a single intron. By comparison, 90% of the 50 tRNA-IPT genes have 6–8 introns. These results suggest that ATP/ADP-IPT genes are probably derived from an intron-free ancestral tRNA-IPT gene.

We further explored the motif composition of IPT proteins from four representative flowering plants (Figs. 3 and S1). Fifteen conserved motifs were identified. All three types of IPTs, Class I and Class II tRNA-IPTs and ATP/ADP-IPTs, show conserved motif structures among the members of every type. Motifs 11, 13, and 15 and motif 14 could be specifically detected in the Class I and Class II tRNA-IPTs, respectively, while no particular motif could be identified in the ATP/ADP-IPTs. The Class II tRNA-IPT proteins demonstrate a similar motif composition to the ATP/ADP-IPTs rather than the Class I tRNA-IPTs. In fact, the consensus motif structure of the ATP/ADP-IPTs and the Class II tRNA-IPTs is the same, except for motifs 12 and 14, providing direct evidence to support our above-described hypothesis that ATP/ADP-IPT genes are derived from the Class II tRNA-IPTs.

Differences in the tissue/organ expression patterns of tRNA-IPTs and ATP/ADP-IPTs

To investigate the differences in the expression patterns of tRNA-IPTs and ATP/ADP-IPTs, we analyzed RNA-seq data from the basal angiosperm A. trichopoda and the eudicot woodland strawberry. In A. trichopoda, AmIPT2 and AmIPT4 belong to the ATP/ADP-IPT subfamily. The mRNA level of AmIPT4 was low in vegetative organs and meristems and undetectable in female buds, while AmIPT2 was highly expressed in vegetative organs and weakly expressed in meristems and female buds (Fig. S2). In Fragaria vesca, five genes (FveIPT1, FveIPT3–6) belong to the ATP/ADP-IPT subfamily. FveIPT1, FveIPT3, and FveIPT4 were highly expressed in the various stages in carpel and anther. FveIPT5 was highly expressed in ghost and in some stages of cortex and pith, and the mRNA level of FveIPT6 was high in style and some stages of pith (Fig. 4). These different expression patterns indicate that functional diversification of the ATP/ADP-IPT genes after their expansion occurred in eudicots.

By comparison, the three genes (AmIPT1, AmIPT3, and AmIPT5) in A. trichopoda and two genes (FveIPT2 and FveIPT7) in F. vesca, which belong to the tRNA-IPT gene family, were relatively highly and stably expressed in all tissues, suggesting that tRNA-IPTs have a constitutive expression pattern similar to that of housekeeping genes.
We further used qRT-PCR to compare the differences in the expression patterns of the *F. vesca* ATP/ADP-IPT and tRNA-IPT genes in six stages of fruits (little green, big green, white, preturning, pink, and red) and three vegetative organs (leaves, immature roots, and mature roots, Fig. 5). Similar to the results of the above transcriptomic analyses, ATP/ADP-IPT genes showed quite diversified expression patterns. Although the transcript levels of *FveIPT1*, *FveIPT5*, and *FveIPT6* gradually increased during fruit ripening, *FveIPT1* exhibited higher expression levels in leaves, and *FveIPT5* and *FveIPT6* were highly expressed in immature roots. The expression of *FveIPT3/4* was undetectable in all these samples (<5 × 10^{-5}). Meanwhile, the tRNA-IPT genes were constitutively expressed in different developmental stages of fruits, leaves, and roots. We further compared the variation amplitude of *FveIPT* gene expression in different organs. The variation amplitude of the expression of the tRNA-IPT genes (~2-fold) was substantially smaller than that of the ATP/ADP-IPT genes (30- to 130-fold). Therefore, the qPCR results showed that the ATP/ADP-IPT genes have quite diversified expression patterns, while the expression of the tRNA-IPT genes is relatively consistently high throughout the plant.

**Differences in the environmental stress responses of tRNA-IPTs and ATP/ADP-IPTs**

CKs produced by IPT enzymes also play important roles in the regulation of plant responses to environmental stresses. We further investigated the expression profiles of the ATP/ADP-IPT and tRNA-IPT genes under salt, dehydration, hot, and cold stress conditions (Fig. 6). Among the ATP/ADP-IPT genes, the expression of *FveIPT1* was significantly decreased (>4-fold and P < 0.001) after salt stress, and the expression of *FveIPT5* was significantly decreased after salt (>7-fold) and drought stress at 8 h (>3-fold). The transcription of *FveIPT6* was significantly decreased by more than 2–3-fold after salt, dehydration, and cold stress and was increased by 2-fold under heat stress. *FveIPT3/4* was undetectable after all four types of stress (<4 × 10^{-5}). For the tRNA-IPT genes, the transcription of *FveIPT7* was reduced by ~2-fold after...
salt and dehydration stress (Fig. 6). The changes in ATP/ADP-IPT gene expression levels were drastic in response to environmental stresses, but much smaller changes were observed in the expression levels of tRNA-IPT genes under the same stress conditions. These results show that ATP/ADP-IPT genes may be involved in plant responses to environmental stresses, whereas tRNA-IPTs are minimally involved in stress responses.

Discussion

IPT enzymes catalyze the first step of CK biosynthesis. ATP/ADP-IPTs and tRNA-IPTs use different substrates and produce distinct biologically active CKs in flowering plants. In the present study, we performed comprehensive analyses of the differences between these two types of IPT genes in terms of their taxonomic distribution, copy number variation, evolutionary history, duplication mechanism, tissue/organ expression, and environmental stress responses (Table 3). Based on the results here and those previously published by others, we hypothesize that ATP/ADP-IPTs are more important in regulating organ development and environmental stress responses, while tRNA-IPTs mainly play a housekeeping role in angiosperms.

Different origins of ATP/ADP-IPTs and tRNA-IPTs

According to phylogenetic analyses, Lindner et al. hypothesized that Class I tRNA-IPTs have a mitochondrial origin and that Class II tRNA-IPTs are derived from a duplication of a Class I tRNA-IPT gene in the plant lineages. Nishii et al. later proposed that Class II tRNA-IPTs originated from eukaryotic genes. However, based on a broad and balanced sampling of species from major lineages of all three domains of life (eukaryotes, bacteria, and archaea), our analyses indicate that the coexistence of Class I and II tRNA-IPTs can be traced back to the last common ancestor of eukaryotes. Nonplant eukaryotes, except D. discoideum, were found to contain both Class I and Class II tRNA-IPT homologs for the first time in this
study. The reason that Nishii et al.\textsuperscript{27} did not observe this is likely because of their limited sampling of nonplant eukaryotic species. These nonplant species belong to different supergroups that are considered to have diverged from the base of the eukaryotic tree\textsuperscript{32}; thus, our results show that the two classes of tRNA-IPTs coexisted in the last eukaryotic common ancestor (LECA).

In this study, we identified two tRNA-IPT homologs in archaeal species for the first time. tRNA-IPTs have been previously reported to be widely distributed in other kingdoms but not in archaea\textsuperscript{19,26,31}. Although it is unclear whether the archaeal tRNA-IPTs are functional, their identification reveals that tRNA-IPT sequences are distributed throughout all three kingdoms and suggests the possibility of an archaeal origin for tRNA-IPTs. Our phylogeny indicates that bacterial or archaeal species contain only one type of tRNA-IPT homolog (Fig. 1 and Table S1). The Class I tRNA-IPTs are likely to have a closer relationship with the homologs in proteobacteria and proteobacteria-related bacteria, whereas the Class II tRNA-IPTs are more closely related to other bacterial groups and the two archaeal species. Eukaryotes are believed to have resulted from the initial endosymbiotic event involving α-proteobacterial and archaeal cells\textsuperscript{28}. Therefore, we propose that Class I tRNA-IPTs may be derived from the α-proteobacteria that were involved in the initial endosymbiotic event. The Class II tRNA-IPT gene in the LECA appears to have two alternative origins; it is derived from either the archaeal ancestor of eukaryotes or a nonproteobacteria ancestral homolog that was introduced into the LECA via horizontal gene transfer. Because tRNA-IPTs are present in the latest common ancestor of eukaryotes, these tRNA-IPTs are ‘ancient’ CK biosynthesis genes in angiosperms.

Our phylogenetic and motif analyses indicate that ATP/ADP-IPT genes are derived from Class II tRNA-IPTs. Most ATP/ADP-IPT genes contain no or only one intron, whereas Class II tRNA-IPTs contain many introns. This difference in gene structure suggests that either the original ATP/ADP-IPT gene was generated via a retroposition duplication of a Class II tRNA-IPT gene or that intron loss occurred soon after its emergence. Intronless daughter gene(s) and sequences of short direct repeats are the two main molecular features of retroposition\textsuperscript{33,34}. We searched the ~100 kb regions upstream to downstream of all the ATP/ADP-IPT genes in Arabidopsis but did not

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5}
\caption{Expression profiles of the FveIPT genes in roots, leaves, and different early-stage fruits. Transcript expression was normalized to the expression of the GAPDH gene. The mean ± s.d. of three biological replicates is presented. The different lowercase letters above the bars indicate significant differences (\(\alpha = 0.05\), LSD) among the different tissues. Three biological replicates and three technical replicates were performed for each data point.}
\end{figure}
find any featured short direct repeat sequences. Consequently, although Class II tRNA-IPTs account for the origin of ATP/ADP-IPT genes, the duplication mechanism is still unclear. Nevertheless, because the evidence from previous studies and the current study clearly demonstrates that the ATP/ADP-IPT genes emerged in flowering plants, we call them ‘non-ancient’ CK biosynthetic genes relative to the tRNA-IPTs, which we call ‘ancient’ CK biosynthetic genes. In addition, ATP/ADP-IPTs were previously proposed to only be present in flowering plants. In our phylogeny of IPTs from all three domains of life, four of the five gymnosperm IPTs branch with tRNA-IPTs, while the rest (PalIPT3) phylogenetically clusters with angiosperm ATP/ADP-IPTs (Fig. 1). Although the support for this clustering is not great, this close relationship suggests that PalIPT3 is probably an ATP/ADP-IPT gene. ATP/ADP-IPTs are responsible for the biosynthesis of tZ-type CKs. It has been observed that tZs, rather than cZs, are predominant in vegetative shoots/leaves in most gymnosperms and angiosperms. By comparison, in seedless plants, whose IPTs all phylogenetically branch with tRNA-IPTs, cZs are the most abundant CKs. The general CK composition of gymnosperms, which is similar to that of angiosperms but distinct from that of seedless plants, suggests that gymnosperms have a similar IPT gene composition to that of angiosperms; that is, gymnosperms may already contain ATP/ADP-IPT genes. Future investigations on the biochemical characteristics, including preferred substrates and products, of gymnosperm IPT proteins are needed to functionally confirm that ATP/ADP-IPT genes originate from gymnosperms.

Fig. 6 Expression profiles of FveIPT genes in response to environmental stresses. The expression levels relative to that of GAPDH were measured by qRT-PCR. Asterisks indicate that the corresponding gene was significantly upregulated or downregulated under the given condition (*p ≤ 0.05). Three biological replicates and three technical replicates were performed for each data point.

tRNA-IPTs and cZ-type CKs likely play housekeeping roles, while ATP/ADP-IPTs and iP/tZ-type CKs may be involved in organ development and stress responses

Based on the following lines of evidence, we hypothesize that in angiosperms, tRNA-IPTs and associated cZ-type CKs mainly play housekeeping roles to maintain basic cellular functions, whereas ATP/ADP-IPTs and their products, the iP-type and tZ-type CKs, are more likely to be involved in the regulation of organ development and stress responses.

First, expression profiling indicates the functional differences between ATP/ADP-IPTs and tRNA-IPTs. Our genomic transcriptome and qPCR analyses of F. vesca, for instance, demonstrated that ATP/ADP-IPT genes display drastic changes at the expression level in various tissues/organs and developmental stages (Figs. 4 and 5). In contrast, tRNA-IPTs are constitutively and relatively stably expressed in all tissues and developmental stages throughout the plant. Similar expression patterns of the ATP/ADP-IPT and tRNA-IPT genes have also been observed in the tissues/organs of Arabidopsis, Z. mays (summarized in Figs. S3 and S4, respectively) and other species. Therefore, the expression patterns of the IPT
genes suggest that \textit{ATP/ADP-IPTs} play regulatory roles in organ development in angiosperms, while \textit{tRNA-IPTs} most likely act as housekeeping genes.

Second, the evolutionary histories of \textit{ATP/ADP-IPTs} and \textit{tRNA-IPTs} in angiosperms support their different roles. Our results demonstrate that \textit{tRNA-IPTs} are conservatively retained as two or three copies in flowering plants, while \textit{ATP/ADP-IPTs} have undergone considerable expansions and are highly variable in gene number (from 2 to 21) among species. Moreover, we found that the \textit{ATP/ADP-IPT} genes (\textit{AmIPT2} and \textit{4}) were rarely expressed in the flower buds of \textit{A. trichopoda} (Fig. S2), an extant basal flowering plant with primitive flower tissues. In contrast, most \textit{ATP/ADP-IPT} genes are highly expressed in different floral tissues or developmental stages in core angiosperms, such as \textit{F. vesca}, \textit{Arabidopsis}, and maize. This discrepancy suggests that the expansion and functional diversification of \textit{ATP/ADP-IPT} genes in eudicots and monocots have contributed to their diverse regulatory roles in floral organs.

Third, the manipulation of the expression of different \textit{ATP/ADP-IPT} and \textit{tRNA-IPT} genes gives rise to different phenotypic variations. The \textit{tRNA-atipt9} single and \textit{tRNA-atipt2/9} double mutant plants of \textit{Arabidopsis} exhibited an overall small and chlorotic phenotype with no obvious alterations in organ differentiation or development. In contrast, the \textit{ATP/ADP-IPT}-mutant plants \textit{atipt3} or \textit{atipt5} had increased numbers of lateral roots and lateral root primordia. The triple mutant \textit{atipt3;5;7} exhibited a decrease in dedifferentiation ability, which resulted in the reduced formation of callus tissues from root explants after wounding. The quadruple \textit{ATP/ADP-IPT} mutant \textit{atipt1;3;5;7} produced fewer leaves, altered inflorescence, and was unable to develop cambium in the root and stem. Additionally, the overexpression of an \textit{ATP/ADP-IPT} gene resulted in reduced root growth, enhanced shoot differentiation, increased branching, and increased flower number along with abnormal floral organ development. Altogether, the phenotypic changes of these mutant or transgenic plants indicate that \textit{ATP/ADP-IPTs} regulate organ development in angiosperms but that \textit{tRNA-IPTs} do not.

Fourth, the iP/tZ-type and cZ-type CKs produced by \textit{ATP/ADP-IPTs} and \textit{tRNA-IPTs}, respectively, have been shown to be differentially involved in developmental processes in flowering plants. Higher levels of iP and tZs than cZs have been detected in apical meristems, fertilized embryos and other tissues with high dedifferentiation and differentiation activities. Furthermore, tZs were much more potent than cZs in an assay of callus and shoot development.

| Table 3 | Comparison of \textit{ATP/ADP-IPTs} and \textit{tRNA-IPTs} in flowering plants |
|----------|----------------------------------------------------------|
| **Substrate** | ADP or ATP (non-ancient) |
| **Product** | iP and tZs |
| **Gene structure** | Intron-less (non-ancient) |
| **Origin** | Last common ancestor of flowering plants |
| **Copy number** | 2–21 (average 8.13) |
| **Expression pattern** | Tissue-specific |
| **Abiotic stress responses** | Highly responsive |
| **Mutant phenotypes** | Increased lateral root primordia and lateral root numbers, decreased numbers of vascular bundles and cells, reduced shoot apical meristem size, reduced inflorescence, inability to form cambium, higher tolerance to drought or salt stress |
| **Overexpression phenotypes** | Enhanced shoot branching and increased floral numbers along with abnormal floral organ development |
| **Effects of exogenous application** | Effects of exogenous iP/tZ-type cytokinins: promotion of callus formation, shoot initiation, lateral root formation, and the development of vascular cambium, shoot apical meristem, inflorescence stems, and ovules |
| **Proposed roles in angiosperms** | Regulating organ development and abiotic stress responses |

\textsuperscript{a}Based on the analyses in this study

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It has been shown that the endogenous content of \( tZs \) was substantially elevated within 12 h after the wounding of root explants, which reflected a similar but earlier increase in comparison with the expression of core cell cycle regulator genes key for callus formation\(^{15,16,41,42}\). A decrease in \( tZs \) and iP\( s \) in the \( ATP/ADP-IPT \) mutant \( atipt3;5;7 \) resulted in reduced callus formation, while an increase in \( tZs \) and iP\( s \) by the over-expression of \( Agrobacterium \) \( IPT \) (AMP-IPT) dramatically enhanced the production of callus and shoots\(^{43,44}\). Additionally, \( tZs \) and iP\( s \) have been shown to be important in regulating the development of shoot apical meristem, vascular cambium, lateral roots, inflorescence stems, and ovules\(^{14,21,22}\). On the other hand, few changes in \( cZ \) levels were detected during wound-induced callus formation, indicating that endogenous \( cZs \) were unlikely to play a significant role. The effects of the overproduction of \( cZs \) or prenylated tRNAs remain unknown, as there has been no report on \( tRNA-IPT \) overexpression so far. However, the exogenous application of \( cZs \) are relatively ineffective in stimulating shoot and other organ development under in vitro conditions\(^{15,16,41,42}\). \( cZ \)-deficient \( tRNA-ipt \) mutant plants of \( Arabidopsis \) are chlorotic and show much reduced plant sizes\(^{14,20}\), suggesting that \( cZ \)-type CKs may be essential for maintaining the basic functions of cells. Taken together, the results suggest that \( ATP/ADP-IPTs \) and the associated iP\( s \) and \( iZ \)-type CKs function as important regulators of angiosperm organ development, whereas \( tRNA-IPTs \) and the associated \( cZ \)-type CKs play basic housekeeping roles in angiosperm cells.

Fifth, \( ATP/ADP-IPTs \) and \( tRNA-IPTs \) catalyze different biochemical reactions that produce two distinct types of CKs, the iP\( s/tZs \) and the \( cZs \), respectively\(^{14}\). \( ATP/ADP-IPTs \) catalyze the rate-limiting step of the de novo biosynthesis of CKs, directly generating iP\( s \) and \( iZ \) nucleotides or nucleosides that are subsequently converted into their active free-base forms\(^{19}\). By comparison, the \( tRNA-IPTs \) in angiosperms primarily catalyze the isopentenylation of adenine in tRNA to produce \( cZs \) from the degradation of prenylated tRNAs\(^{14,19}\). Although the function of prenylated tRNA remains unclear in plants, studies in bacteria, yeast, and mammalian cells have shown that tRNA prenylation is important for translational efficiency and fidelity\(^{45}\) by preventing frameshifts and the nonsense suppression of the codon UAA\(^{40,46}\). Moreover, despite the relatively high expression of \( tRNA-IPTs \) throughout the plant, the content of endogenous \( cZs \) is generally low\(^{16}\). The application of \( cZ \) to \( 35S:AtCKX7 \) plants that had reduced \( cZ \) levels could correct their shorter root phenotype, whereas the application of \( cZ \) to \( tRNA-atipt2;9 \) plants could not\(^{20}\). These results indicate that the reduced root growth observed in \( tRNA-atipt2;9 \) mutants may not be due to the loss of \( cZ \) production but more likely results from the loss of tRNA prenylation, suggesting that another housekeeping function of \( tRNA-IPTs \) is to maintain translational accuracy, which is essential for normal cellular activities.

Sixth, similar to their roles in organ development, angiosperm \( ATP/ADP-IPTs \) have also diversified in response to environmental stresses. Our results show that the expression levels of all three expressed \( ATP/ADP-IPT \) genes in \( F. \) \( vesca \) are substantially reduced under heat, cold, drought, or salt stress (Fig. 6). Studies in other angiosperms have observed similar findings. In rice, salinity suppressed, whereas cold or drought stress enhanced the expression of most \( ATP/ADP-IPT \) genes\(^{37}\). In \( Arabidopsis \), the expression patterns of \( ATP/ADP-IPT \) genes under stress conditions were also differentially regulated in shoots and roots\(^{47}\). Moreover, the \( iZ \)-type CKs that are produced by \( ATP/ADP-IPT \) vary upon environmental stress, depending on the type and intensity of the stress treatment\(^{40}\). In contrast, \( tRNA-IPT \) genes display relatively little variation in their expression level when plants are subjected to environmental stresses (Fig. 6), as shown in previous studies\(^{37,40}\). Although the elevation of \( cZ \) levels has been found under stress conditions, it is generally recognized that \( cZs \) are only a byproduct of increased prenylated tRNA turnover but are not produced from the expression of the \( tRNA-IPT \) genes\(^{40,48}\). These observations indicate that \( tRNA-IPTs \) are more likely to play a housekeeping role, whereas \( ATP/ADP-IPTs \) make an important contribution to the regulation of plant responses to environmental stresses.

Additionally, along with a reduction in the expression levels of \( ATP/ADP-IPTs \) under abiotic stress conditions, \( ATP/ADP-IPT \) mutant plants that contain deficient iP\( s \)/iZ-CK levels but little changed \( cZ \) levels, such as the quadruple \( atipt1;3;5;7 \) \( Arabidopsis \) mutant, display increased tolerance to drought and salt stress\(^{23}\). There have been few experiments that have shown the effects of altered \( cZ \) content on plant responses to abiotic stress. However, the overexpression of \( AtCKX \) isoforms, which led to substantial decreases in iP\( s \)/iZ-CKs but either largely reduced or unchanged \( cZ \) levels, resulted in improved levels of tolerance to drought or salt stress\(^{25}\). Therefore, compared with iP\( s \)/tZ\( s \), variations in \( cZ \) content appear to have little effect on plant tolerance to abiotic stress. It has been suggested that \( cZs \) produced by \( tRNA-IPTs \) are more involved in the maintenance of a basal level of CK activity under growth-limiting conditions\(^{15,40}\). Accordingly, it is more likely that the elevation of \( cZ \) levels in stressed conditions is associated with their housekeeping role in plant growth and development, whereas iP\( s \)/iZ-type CKs produced by \( ATP/ADP-IPTs \) play an important role in the regulation of plant adaptation to environmental stresses.

In summary, based on our results that are presented in this manuscript and previously published data, we
hypothesize that tRNA-IPTs (ancient CK biosynthesis genes) and the associated cZs play a housekeeping role, whereas ATP/ADP-IPTs (non-ancient CK biosynthetic genes) and the associated iP/iZ-type CKs play regulatory roles in organ development and stress responses in angiosperms. An accompanying paper in this issue provides additional evidence to support this hypothesis\(^59\). It is our hope that this hypothesis will stimulate more interest in elucidating the origins, evolutionary significance, and roles of different types of IPTs (tRNA-IPTs vs. ATP/ADP-IPTs) and the associated different types of CKs (iP/iZ vs. cZ types) in regulating organ development and responses/adaptation in angiosperms.

**Materials and methods**

**Homolog identification**

A total of 59 whole-genome-sequenced species sampled from major lineages of all three domains of life (eukaryotes, bacteria, and archaea, Table S1) were first examined for the presence of IPT genes. To explore the evolution of IPT genes in land plants, 19 sequenced species belonging to every major lineage of land plants (Table S2) were next sampled and analyzed. The complete genomic sequences and corresponding annotation information for all these species were downloaded from the JGI and NCBI databases. The hidden Markov model profile of the IPPT domain (PF01715) was downloaded from the Pfam database\(^50\), which was used as a query to search for homologous sequences in the proteome data sets. Sequences with an expected value (E-value) \(< 10^{-4}\) were considered candidates. The chromosomal locations of all candidates were verified to remove redundant sequences. Short proteins with lengths \(<100\) aa were removed\(^51\). We further confirmed the presence of the IPPT domain in each candidate using the PFAM (http://pfam.xfam.org/search)\(^50\) and SMART databases (http://smart.embl-heidelberg.de\(^52\)) with an E-value cut off \(<1e^{-10}\). For archaea, TBLASTN was first used to find potential hits for the IPPT domain under the threshold of 1.0. For the significant hits, the corresponding amino acid sequences of the high-scoring segment pairs (HSPs) were retrieved to assess the presence or absence of the IPPT domain via the PFAM and SMART databases.

**Phylogenetic analyses**

The full-length amino acid sequences of all identified IPTs were aligned using ClustalX 2.0\(^53\). The best-fit model for protein evolution was selected using the Model-Generator program\(^54\). A phylogenetic tree of the IPT family was constructed via the maximum-likelihood (ML) method using PhyML (version 3.0) software\(^55\) with the JTT evolutionary model. The tree topology was reconstructed using the best of nearest-neighbor interchange (NNI) and subtree pruning and regraphing (SPR) methods\(^55\). Branch supports were estimated using an approximate likelihood ratio test with a Shimodaira–Hasegawalike procedure\(^55\). The phylogenetic trees were visualized in FIGTREE.

**Gene structure, protein motif, and synteny analyses**

The relative intron positions in the IPT genes were extracted from the annotated whole-genome data sets of 19 land plants. The gene structure was viewed in the online software Evolview (http://www.evolgenius.info/evolview/). MEME\(^56\) was used to discover the conserved motifs in IPT proteins from the four representative flowering plants, A. trichopoda, Zea mays, A. thaliana, and F. vesca. The parameters were set as follows: the maximum number of motifs, 15; minimum motif width, 6 aa; maximum motif width, 50 aa.

Syntenic analyses of the 15 flowering plant genomes were conducted locally using a method similar to that developed for the PGDD (http://chibba.agtec.uga.edu/duplication)\(^57\). BLASTP was used to search for potential homologous gene pairs (E<1e\(^{-10}\), top five matches) across multiple genomes. These homologous pairs were then used as the input for MCScanX to identify the syntenic regions\(^59,60\). MCScanX was further used to identify the IPT genes resulting from WGD/segmental, tandem, proximal, and dispersed duplications (http://chibba.agtec.uga.edu/duplication/index/downloads).

**Transcriptome data analyses**

Transcriptome data from different tissues and developmental stages of F. vesca\(^59,60\) and A. trichopoda were downloaded from the SGR (http://bioinformatics.towson.edu/strawberry/) and NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA212863) databases, respectively. The reads per kilobase per million (RPKM-mapped reads) values were directly retrieved from the website, and the expression levels for the IPT genes were plotted on a log scale.

**Plant growth conditions, stress treatments, and material collection**

All plant materials were collected from a seventh-generation inbred line of F. vesca, Ruegen (kindly provided by Janet Slovin). For the strawberry fruit collection, strawberry plants were grown in 10 cm × 10 cm pots in a greenhouse with a 12 h photoperiod at 25 °C with 65% relative humidity. Strawberry fruits at the little green (2–4 days after anthesis), big green (8–10 days after anthesis), white, preturning, pink (slight pink flesh and red seeds) and red stages (2–3 days after the pink stage) as well as leaves, immature roots, and mature roots were collected prior to immediate submersion in liquid nitrogen. The tissues from at least three samples were combined to form one biological replicate, and each tissue type utilized three biological replicates.
For the environmental stress experiments, sterile strawberry seedlings were grown in magenta boxes for 2 months in a growth chamber with a 16 h photoperiod at 22 °C and 3000 lx. For the heat shock treatment, the seedlings were transferred to a growth chamber at 38 °C and 3000 lx and were collected at 1, 3, 4 (3 h heat shock and 1 h recovery at 22 °C) and 8 h (3 h heat shock and 5 h recovery at 22 °C). Prior to the salinity stress treatment, the seedlings were transferred to 1/2 MS liquid media and cultivated with gentle agitation (100 rpm). After 12 h, the seedlings were transferred to 1/2 MS liquid media supplemented with 150 mM sodium chloride. For drought treatment, the seedlings were placed on filter paper under dim light at 22 °C with 65% relative humidity. For cold treatment, the seedlings were transferred to a growth chamber set at 4 °C (in the dark). Salinity-stressed, cold-treatment, the seedlings were transferred to a growth chamber set at 22 °C (in the dark). Drought-stressed, cold-stressed, and dehydration-stressed seedlings were collected at 1, 3, and 8 h after the beginning of the treatment. All collected plant materials were immediately submerged in liquid nitrogen prior to RNA processing.

Quantitative real-time PCR (qRT-PCR) analysis

A modified CTAB method was used for RNA isolation from all the samples mentioned above. The isolated RNAs were treated with DNase I and used for cDNA synthesis using the Primerscript RT Reagent Kit with gDNA Eraser (Takara). qRT-PCR was performed using SYBR Premix Ex Tag (Takara) with the cDNA as the template. The IDT website (http://sg.idtdna.com/site) was used to design the qRT-PCR primers for the FveIPT genes (Table S3). The sequence similarity between FveIPT3 and FveIPT4 is >90%; thus, we used a single primer set to investigate the combined expression of both genes. The results were analyzed using the $\Delta\Delta CT$ method with GAPDH as the control locus. Three biological and three technical replicates were analyzed.

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Authors’ contributions

Y.L. and J.D. designed the experiments; X.W. and S.L. performed the experiments and data analyses; D.L. participated in the data analyses; J.D., X.W. and Y.L. wrote the manuscript; Y.L., L.G. and R.M. edited and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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