Cytokinins are a group of phytohormones that regulate various aspects of plant growth and development, such as maintenance of stem cell systems in shoots and roots (Kurakawa et al., 2007; Müller and Sheen, 2008; Gordon et al., 2009; Zhao et al., 2010; Tokunaga et al., 2012), nodule organogenesis (Murray et al., 2007; Tirichine et al., 2007; Kouchi et al., 2010), shoot and root vascular development (Higuchi et al., 2004; Hutchison et al., 2006; Mähönen et al., 2006; Ishida et al., 2008; Kondo et al., 2011). This hormone is also synthesized by some phytopathogenic bacteria, such as Agrobacterium tumefaciens, Rhodococcus fascians, and Pseudomonas savastanoi, and consequently plays a key role for plant tumorigenesis (Morris, 1986; Goethals et al., 2001; Pertry et al., 2009). A. tumefaciens infection of plant cells induces the formation of “crown gall” tumors. This neoplastic morphogenesis is induced by the expression of genes encoded within the T region of Ti plasmids (Van Larebeke et al., 1974). Upon infection, the T region is introduced into host plant cells by a type IV secretion system (Alvarez-Martinez and Christie, 2009) and integrated into the nuclear genome (Chilton et al., 1977; Zhu et al., 2000; Zupan et al., 2000). The T region contains genes involved in the biosynthesis of cytokinins, auxin, and opines, and the genes are expressed by the host plant transcription/translation machinery (Suzuki et al., 2009). Aberrant cell proliferation caused by the overproduction of cytokinins and auxin in infected cells results in tumor formation (Escarob and Dandekar, 2003).

Cytokinins are adenine derivatives that carry an isoprene-derived side chain at the N6 terminus (Mok and Mok, 2001; Sakakibara, 2006). The first step of cytokinin biosynthesis is catalyzed by adenosine phosphate-isopentenytransferase (IPT). The T regions of Ti plasmids commonly contain an IPT gene (tmr; Akiyoshi et al., 1984; Barry et al., 1984). Tmr catalyzes the condensation of AMP with 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) or dimethylallyl diposphate (DMAPP) to form trans-zeatin (tZ) riboside 5′-monophosphate or N6-(Δ2-isopenteny)adenine.
(iP) riboside 5'-monophosphate, respectively, which are precursors of cytokinins (Akiyoshi et al., 1984; Barry et al., 1984; Sakakibara et al., 2005; Sakakibara, 2006). Tmr is localized in the plastids of host plants despite lacking a typical transit peptide at the N terminus. The enzyme modifies cytokinin biosynthesis by recruiting HMBDP, an intermediate product of the methylerithiol phosphate pathway in plastids, to directly produce tZ riboside 5'-monophosphate without CYP735A-mediated trans hydroxylation (Takei et al., 2004; Sakakibara et al., 2005). Despite the similar affinity of Tmr for HMBDP and DMAPP in vitro (Sakakibara et al., 2005; Sugawara et al., 2008), crown galls and Tmr-overexpressing transgenic plants almost exclusively contain tZ and its conjugates (tZ-type cytokinins; Morris, 1986; Faiss et al., 1997; Sakakibara et al., 2005), implying an unknown mechanism for preferential HMBDP usage by Tmr in vivo. In addition to tmr, nopaline-type Ti plasmids encode another IPT gene (tzs) within the virulence region, which is induced by plant phenolic compounds (John and Amasino, 1988). The Tzs and Tmr proteins are structurally similar, with 51.3% identity at the amino acid level, whereas higher plant IPTs have lower similarity to Tmr (approximately 20% identity). In vitro studies indicate that both Tzs and Tmr utilize HMBDP and DMAPP with similar affinity (Krall et al., 2002; Sugawara et al., 2008). Tzs is localized on the bacterial cell surface and may contribute to the interactions of A. tumefaciens with the host plants via cytokinin production (Aly et al., 2008).

The two A. tumefaciens IPTs are involved in cytokinin production under spatially and temporally separated conditions. However, the functional specialization of Tmr for the shortcut pathway and the plastid localization of tZ precursor biosynthesis and tumor formation are not well understood. As examined so far, the ability to transform directly HMBDP into trans-zeatin riboside 5'-phosphates (tZRPs) is only found in Tmr, suggesting that Tmr is uniquely specialized for tumorigenesis. A comparison of the biochemical and functional features between Tmr and Tzs should provide a clue about the specialization. In this study, we examined the activity of Tzs as an alternative to Tmr in terms of preferential substrate usage and plastid localization. We also evaluated the tumor-forming ability of genetically modified A. tumefaciens strains whose tmr was replaced with another IPT. Our results strongly suggest that Tmr plastid localization and the preferential usage of HMBDP in vivo are both important for efficient gall formation.

RESULTS
Tmr Uses HMBDP Preferentially in Planta, But Tzs Does Not

Previously, we have shown that Tmr localizes in the plastids of host plant cells and directly produces tZ nucleotide using HMBDP and that transgenic Arabidopsis (Arabidopsis thaliana) plants that overexpress Tmr predominantly accumulate tZ-type cytokinins (Sakakibara et al., 2005). To examine the specialization of Tmr in terms of prenyl donor substrate usage in vivo, we compared postinduction concentrations of tZRPs and N6-(Δ2-isopentenyl)adenine riboside 5'-phosphates (iPRPs) in Arabidopsis plants that were transformed with either tzs or tmr (Sakakibara et al., 2005) under the control of a dexamethasone (DEX)-inducible promoter (Fig. 1; Supplemental Table S1). Induction of Tzs expression by DEX treatment resulted in the production of roughly equal amounts of iPRPs and tZRPs (Fig. 1A). This result contrasts with our observation of a clear difference in iPRP and tZRP synthesis in Tmr-overexpressing Arabidopsis (Fig. 1B; Sakakibara et al., 2005). The overall accumulation of cytokinins in Tzs-overexpressing Arabidopsis plants was substantially lower than in Tmr-overexpressing plants (Fig. 1), even though the substrate affinities of Tmr and Tzs for HMBDP and DMAPP are similar in

Figure 1. Cytokinin accumulation in transgenic Arabidopsis lines overexpressing agrobacterial IPTs. Transgenic Arabidopsis seedlings harboring pTA-Tzs (A; Tzs-ox), pTA-Tmr (B; Tmr-ox), and pTA-7001 empty vector (C; Control) were grown for 18 d on MGRL-agar plates and then sprayed with 30 μM DEX. After 0, 4, 7, and 24 h, the whole seedlings were harvested and cytokinin contents were measured. Only the results of iPRPs and tZRPs are presented. Error bars represent SD of three biological replicates. FW, Fresh weight. The complete data set is presented in Supplemental Table S1.
vitro (Sakakibara et al., 2005; Sugawara et al., 2008), and the $k_{cat}/K_m$ values of Tmr are rather lower than those of Tzs (Supplemental Fig. S1). These results suggest that Tmr preferentially uses HMBDP in planta but Tzs does not and that Tmr has higher catalytic activity than Tzs in vivo.

Plastid Import Is Largely Limited to Tmr

Translational fusions of Tzs were made with GFP at the C terminus (Tzs-GFP) under the control of the cauliflower mosaic virus 35S promoter and introduced into Arabidopsis cells by particle bombardment (Fig. 2). When the fusion gene was transiently expressed in leaf and root cells, the GFP fluorescence of Tzs-GFP was distributed in plastids, nuclei, and cytosol (Fig. 2, A–C) whereas Tmr-GFP was restricted to plastids (Fig. 2, D–F), as reported previously (Sakakibara et al., 2005). To further obtain information about localization, chloroplasts were enriched from stable transformants of Arabidopsis shoots in which Tzs expression was induced by DEX treatment for 24 h, and Tzs polypeptide was detected with anti-Tzs antibody in immunoblot assays. Very little Tzs polypeptide was recovered in the chloroplast-enriched fraction, but plastidic Gln synthetase concentrations in the chloroplast fraction proteins, used as a control, were comparable to total leaf proteins (Fig. 2G). It should be noted that Tmr polypeptide had been recovered in the chloroplast-enriched fraction using the same experimental method (Sakakibara et al., 2005). This result indicates that the ability of Tmr to be imported into plastids is not well conserved in Tzs.

Obligatory Plastid Localization of Tzs Does Not Change Substrate Use Preference

To understand the relationship between Tmr plastid localization and its preferential use of HMBDP, a fusion protein construct was made between the plastid-target transit peptide and Tzs at the N terminus (TP-Tzs). Because of this arrangement, it was assumed that TP-Tzs was translocated to plastid stroma via the Toc/Tic system (Soll and Schleiff, 2004; Inaba and Schnell, 2008). Since A. tumefaciens infected galls are usually induced in nonphotosynthetic organs, we used the nonphotosynthetic-type ferredoxin III (Fd III; Hase et al., 1991). Because the cleavage site of Fd III is between Met-55 and Ala-56 (Hase et al., 1991; Suzuki et al., 1991), mature polypeptide from TP-Tzs in plastid was expected to have an extra Ala residue at the N terminus. To test whether this extra Ala residue introduced at the N terminus of Tzs affects the catalytic activity of TP-Tzs, we expressed the recombinant proteins with a poly-His tag in Escherichia coli, purified the protein, and checked the enzyme activity in vitro. The poly-His-tagged recombinant Tzs protein with N-terminal Met-Ala has catalytic activity for HMBDP and DMAPP comparable to wild Tzs, but the activity of TP-Tzs is not detectable (Supplemental Fig. S2).

When the chimeric TP-Tzs-encoding gene was translationally fused to GFP at the C terminus (TP-Tzs-GFP) and transiently expressed in Arabidopsis mesophyll cells, GFP fluorescence was only observed in chloroplasts (Fig. 3A). We generated stable transgenic Arabidopsis lines overexpressing the TP-Tzs fusion
under the control of the DEX-inducible promoter. After the induction of TP-Tzs expression by DEX treatment, accumulation of the Tzs polypeptide in leaf chloroplasts and root plastids was examined by immunoblots. Although the accumulation of mature Tzs protein in leaves was higher in TP-Tzs-ox in comparison with Tzs-ox, the polypeptides were not present in high concentrations in the chloroplast-enriched fractions of two independent transgenic lines (TP-Tzs-ox #1 and #2; Fig. 3B). In the roots of TP-Tzs-ox (TP-Tzs-ox #2), Tzs polypeptides were extracted at more elevated levels in the plastid-enriched fraction. The signal ratio of total to plastid-enriched fractions in Tzs was comparable to plastidic Gln synthetase (Fig. 3C, right panels). These results suggest that TP-fused Tzs is efficiently transported into plastids in TP-Tzs-ox line plants, especially in the nonphotosynthetic organs.

We harvested the transgenic Arabidopsis seedlings under the same conditions to assay cytokinin (Fig. 3D; Supplemental Table S2). Although the accumulation of cytokinins was remarkably higher in transgenic Arabidopsis plants expressing TP-Tzs (TP-Tzs-ox) in comparison with Tzs-ox, the major species of cytokinins were all iPRPs in two independent transgenic lines (Fig. 3D). These results suggest that the imported TP-Tzs is processed to form a functional enzyme, but if any Tzs is localized in plastids, it could not preferentially use HMBDP as a prenyl donor.

Comparison of Tumor Formation Efficiency with Tmr and Tzs

To evaluate Tmr specialization for tumorigenesis, we compared tumor formation efficiency among A. tumefaciens strains expressing Tmr, Tzs, or TP-Tzs. We first disrupted tzs within the vir region of pTi-SAKURA in A. tumefaciens MAFF301001 rif to exclude any effects of authentic Tzs-derived cytokinins, and we defined this strain (301001tzs⁻) as the wild type for this study (Table I). The tmr gene on the Ti plasmid was then replaced with tzs or TP-tzs by homologous recombination in the A. tumefaciens 301001tzs⁻ strain, and the genotypes in the recombinant strains were designated as tmr/Tzs and tmr/TP-Tzs, respectively. A recombinant A. tumefaciens strain whose tmr gene was replaced with AtIPT1 was also generated, and the genotype was designated as tmr/AtIPT1. AtIPT1 localizes in plastids and exclusively uses DMAPP as its prenyl donor substrate (Kasahara et al., 2004; Sakakibara et al., 2005). To exclude the possibility of

Figure 3. Forced localization of TP-Tzs into Arabidopsis plastids. A, Distribution of TP-Tzs-GFP fusion protein in an Arabidopsis cell. TP-Tzs-GFP was transiently expressed in Arabidopsis leaf mesophyll cells by particle bombardment. B and C, Immunoblot analysis. B, A transgenic Arabidopsis line harboring pTA-Tzs (Tzs-ox) and two independent transgenic Arabidopsis lines harboring pTA-TP-Tzs (TP-Tzs-ox #1 and #2) were grown for 18 d on MGRL-agar plates and then treated with (+) or without (−) 30 μM DEX for 24 h. Total leaf proteins (Total) and chloroplast-enriched fraction proteins (Chl.) were prepared. Proteins equivalent to 0.2 μg of chlorophyll were subjected to immunoblot analysis. The white arrowhead indicates the mobility of mature Tzs. The less mobile polypeptide in TP-Tzs-ox #1, indicated with the black arrowhead, is probably unprocessed precursor. C, Arabidopsis lines Tzs-ox and TP-Tzs-ox #2 were first grown on MGRL-agar plates for 24 d with 12 h of light/12 h of dark and then hydroponically grown with MGRL salt for 18 d with 18 h of light/6 h of dark. After treatment of the roots with (+) or without (−) DEX, total root proteins (Total) and the plastid-enriched fraction proteins (Pl.) were prepared. Six micrograms of protein for Tzs-ox and 4 μg for TP-Tzs-ox were subjected to immunoblot analysis. GS₁ and GS₂, Cytosolic and plastidic Gln synthetase (GS), respectively. D, Cytokinin accumulation in transgenic Arabidopsis lines overexpressing Tzs or TP-Tzs. Transgenic Arabidopsis seedlings of Tzs-ox #1 and #2 (independent lines) and TP-Tzs-ox #1 and #2 were grown for 18 d on MGRL-agar plates; half were subjected to DEX treatment (+) and half were not treated as controls (−). After 24 h, the seedlings were harvested and cytokinin contents were analyzed. Only iPRP and IzRP concentrations are presented. Error bars represent so of three biological replicates. FW, Fresh weight. The complete data set is presented in Supplemental Table S2.
differences in expression efficiency, we replaced tmr with the other gene’s reading frame without any nucleotide insertions or deletions (for details, see “Materials and Methods”). The recombinant A. tumefaciens strains were introduced into Kalanchoe daigremontiana leaves, and tumor formation was measured 34 d after infection (Fig. 4). In Kalanchoe, the tumorigenesis due to tmr/Tzs was substantially less than with the wild type (Fig. 4A). tmr/TP-Tzs strains induced significantly more tumor formation than strains carrying tmr/Tzs but were still much lower than the wild type. When we replaced tzs with AtIPT1 (tmr/AtIPT1), tumorigenesis increased but still did not reach the wild-type level (Fig. 4A). Because it was difficult to obtain enough soluble protein from tumors for immunoblot analysis, expression of the exogenous genes was confirmed by reverse transcription (RT)-PCR. Exogenous cytokinin biosynthesis genes were all expressed (Supplemental Fig. S3). We also analyzed cytokinin and auxin (indole-3-acetic acid [IAA]) concentrations in the tumors (Fig. 4, B and C; Supplemental Table S3). Since the tumors generated from tmr/Tzs and tmr/TP-Tzs strain infections were too small to analyze individually, we pooled each group. Uninfected Kalanchoe leaves served as the control tissue. Accumulation of IAA was higher in all tumor tissues, suggesting successful A. tumefaciens infection with expression of the introduced auxin biosynthesis genes. IZ-type and iP-type cytokinins were the dominant forms in the wild-type A. tumefaciens and tmr/AtIPT1-infected tumors, respectively (Fig. 4B; Supplemental Table S3). The tmr/Tzs- and tmr/TP-Tzs-infected tumors contained much less cytokinin than the wild type or tmr/AtIPT1 and predominantly accumulated iP types, namely the iPRPs (Fig. 4C). The tmr/TP-Tzs-infected tumors contained significantly higher amounts of iPRPs than tmr/Tzs-infected tumors. These results indicate that the expression of Tmr in Kalanchoe should form the largest tumors, that they predominantly accumulate IZ-type cytokinins, and that the introduction of alternative IPTs does not complement the loss of wild-type capabilities.

To further evaluate tumor formation efficiencies in a natural host-pathogen interaction, we infected rose (Rosa sp. ‘Princess Michiko’) stems with the same set of A. tumefaciens strains and evaluated tumorigenesis by measuring gall weights (Fig. 5). Although there was a wide range in tumor weights, the results were essentially the same as in the Kalanchoe assay (Fig. 5A). Galls induced by 301001tzs (the wild type) were largest, galls induced by tmr/Tzs were the smallest, and those induced by tmr/TP-Tzs and tmr/AtIPT1 were significantly larger than tmr/Tzs-induced tumors but smaller than those induced by the wild type. Consistent with gall formation efficiency, IZ-type cytokinins were predominantly accumulated in wild-type galls but iP-type cytokinins predominated in other galls (Fig. 5, B and C; Supplemental Table S4). These results suggest that Tzs, as with plant IPT, is not sufficient to replace Tmr for IZ production and that cytokinin synthesis by Tmr is a critical aspect of specialization during tumor formation.

**DISCUSSION**

In this study, we demonstrate that Tmr is specialized among IPTs for translocation into plastids, selective usage of HMBDP as its prenyl donor, and highly efficient IZ biosynthesis during tumor formation in A. tumefaciens infected galls. This Tmr-specific property is clearly important for larger tumor formation, because alternative IPTs could not complement the capabilities. Although Tzs was not efficiently translocated into plastids, it is intriguing that the fluorescence of Tzs-GFP chimeric protein was observed in plastids as well as cytosol and nuclei (Fig. 2). Dilution of Tzs polypeptide in the intact chloroplast-enriched fraction denied a tight association of Tzs to the chloroplast envelope membrane and efficient translocation of Tzs into the stroma (Fig. 2G). However, GFP fluorescence distribution implies that plastid translocation function is partially conserved in Tzs. In fact, our experiments suggest that a trace amount of Tzs polypeptide could be translocated into stroma, because a small amount of Tzs polypeptide was detected after treatment with proteases (Supplemental Fig. S4). Given that tzs occurs in the nopaline-type Ti plasmids whereas tmr is common to all Ti plasmids so far examined, it could be hypothesized that tzs is derived from an ancestral tmr by gene duplication. If so, plastid translocation signals have been mostly lost in Tzs. As for selective usage of HMBDP, even when Tzs was imported into plastids, it could not preferentially use the hydroxylated substrate (Fig. 3), indicating that this property is not due to subcellular localization but is an intrinsic property of Tmr. At present, there is no evidence to explain the preferential usage of HMBDP in plastids. The in vitro properties of Tmr tend to be DMAPP-philic: the $k_{cat}/K_m$ for DMAPP is about 10 times higher than that for HMBDP (Supplemental Fig. S1). Therefore, there must be a mechanism to control Tmr use of HMBDP in plastids. One possible

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**Table 1. A. tumefaciens strains used for tumor-forming assays**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Properties</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAFF301001 rif</td>
<td>Rif$^+$</td>
<td>Sonoda et al. (2002)</td>
</tr>
<tr>
<td>301001tzs</td>
<td>tzs::Gmr in MAFF301001 rif; Rif$^+$, Gmr$^+$; used as the wild type</td>
<td>This study</td>
</tr>
<tr>
<td>tmr/Tzs</td>
<td>tmr/Tzs replacement mutant of 301001tzs$^+$; Rif$^+$, Gmr$^+$</td>
<td>This study</td>
</tr>
<tr>
<td>tmr/TP-Tzs</td>
<td>tmr/TP-Tzs replacement mutant of 301001tzs$^+$; Rif$^+$, Gmr$^+$</td>
<td>This study</td>
</tr>
<tr>
<td>tmr/AtIPT1</td>
<td>tmr/AtIPT1 replacement mutant of 301001tzs$^+$; Rif$^+$, Gmr$^+$</td>
<td>This study</td>
</tr>
</tbody>
</table>
mechanism is the formation of an enzyme complex containing Tmr and HMBDP synthase (GcpE; Hecht et al., 2001). Complex formation would make it possible to channel HMBDP directly from GcpE to Tmr. Another possibility is that the distribution of HMBDP and DMAPP might be spatially biased in the plastid stroma, because the chemical properties, such as hydrophobicity, of HMBDP and DMAPP should be...
different. If the spatial distribution of HMBDP and DMAPP is not uniform within the plastid, and if Tmr is colocalized with HMBDP, Tmr could preferentially access HMBDP. Further analysis of the suborganellar localization of Tmr in plastids, and searching for Tmr-interacting proteins, will give us a hint to understand the molecular basis of efficient tZ production for effective tumorigenesis. Given and 5). This result suggests that there is an advantage assays, much larger amounts of tZ-type cytokinins such as pH, substrate concentration, and/or magne-

When Tzs was expressed in Arabidopsis under the control of the same promoter system as Tmr, cytokinin accumulation was consistently lower than with Tmr (Fig. 1). On the other hand, forced translocation of Tzs into plastids substantially increased accumulation in two independent lines (Fig. 3D). A similar effect was observed in Kalanchoe and rose tumors (Figs. 4C and 5C). These results suggest that plastid stroma is more suitable for the catalytic reaction of agrobacterial IPT, such as pH, substrate concentration, and/or magne-

It should be noted that in the tumor formation assays, much larger amounts of tZ-type cytokinins were accumulated in galls expressing Tmr than ip-type cytokinins in galls expressing Tzs or a plant IPT (Figs. 4 and 5). This result suggests that there is an advantage to tZ production for effective tumorogenesis. Given that Tzs has generally lower affinity for cytokinin oxidase (Bilyeu et al., 2001), a cytokinin-degrading enzy-

Amino acid identity between Tmr and Tzs is about 51%, and the differences among the amino acid residues likely determine functional specialization in vivo. The atomic structure of Tzs has been solved (Sugawara et al., 2008). A comparison of Tmr and Tzs at the atomic level could provide a clue about the structural basis for the specialization of Tmr in the host plant cell.

MATERIALS AND METHODS

Plants and Bacteria

Arabidopsis (Arabidopsis thaliana) ecotype Columbia was used for gener-

An 1800-bp fragment containing the transit peptide region plus Ala (Met-1 to Ala-56) was amplified by RT-PCR with oligos (Table I), primers for RT and specific primers TP(Fd)-AAB3-5’ and TP(Fd)-Nco1-3’ for subsequent amplification. The DNA amplicon was cloned into pCR-BluntII-TOPO vector (Invitrogen). To obtain cDNA encoding MA-

Generation of Transgenic Arabidopsis Expressing Tzs and TP-Tzs

The coding region of Tzs was ligated into pTA7001 to yield pTA-Tzs. pTA-

Hormone Measurements

Extraction and determination of cytokinins and IAA from Arabidopsis transgenic lines expressing IPTs or galls were performed as described previously with an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) apparatus (AQUITY UPLC System/Quattro Ultima Pt; Waters) with an octadeccsylsil column (AQUITY UPLC BEH C18, 1.7 μm, 2.1 × 100 mm; Waters; Kojima et al., 2009).

Observation of Intracellular Distribution with GFP Fusion Proteins

The coding region of Tzs was ligated into the sGFP (S65T) vector to yield 35Sgfp-Tzs. The 35Sgfp-Tzs, 35Sgfp-TP-Tzs, and 35Sgfp-Tmr (Tmr-GFP; Sakakibara et al., 2005) constructs were introduced into leaf epidermal cells or root cells of 2-week-old Arabidopsis seedlings by particle bombardment (Bio-Rad; PDU-1000/He). Transient expression was observed by confocal laser scanning fluo-

Western-Blot Analysis

About 400 mg of Arabidopsis seedling tissues was homogenized in 2 volumes of ice-cold extraction buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.5). Intact chloroplasts were isolated from Arabidopsis seedlings as described previously (Jackson et al., 1998). Root plastids were enriched as described previously (Emes and England, 1986; Sakakibara et al., 1992a). The proteins were separated by SDS-PAGE followed by western blotting. Polyclonal anti-

Tzs Antibodies

Monoclonal antibodies against Tzs were prepared by Kohjin Bio.

IPT Activity Assay

Recombinant Tzs, MA-Tzs, and TP-Tzs, which has a His, tag at the N ter-

DNA Primers

Primer sequences used in this study are given in Supplemental Table S5.

Construction of TP-Tzs Expression Plasmids

To generate chimeric TP-tzs, which has the transit peptide of maize (Zea mays) Fd III (Hase et al., 1991) at the N terminus of tzs, cDNA encoding the transit peptide region plus Ala (Met-1 to Ala-56) was amplified by RT-PCR with oligos (Table I), primers for RT and specific primers TP(Fd)-AAB3-5’ and TP(Fd)-Nco1-3’ for subsequent amplification. The DNA amplicon was cloned into pCR-BluntII-TOPO vector (Invitrogen). To obtain cDNA encoding MA-

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Ueda et al.
Homologous Recombination of tmr

In order to generate A. tumefaciens mutants, the SacI-based gene-replacement strategy was employed using pK18mobSacB vector (Schäfer et al., 1994). To disrupt tmr in the vir region of Tl plasmid pTi-SAKURA, a gentamycin-resistant gene cassette (Gm') was made by PCR with pUC19Gm (Yamamoto et al., 2009) as the template and primers Gm MluI and Gm MluI 3' and then cloned into pCR-BluntII-TOPO. A 3.7-kb DNA fragment containing tmr and its upstream and downstream 1.5-kb flanking regions was amplified by PCR with pTi-SAKURA and primers Pre-Tzs and Post-Tzs and then cloned into pCR-BluntII-TOPO to yield pTOPO-Tzst-. After confirmation of the nucleotide sequences, a MluI fragment of Gm' was ligated into the MluI site of pTOPO-Tzs+ to yield pTOPO-Tzst+. The BmiII/Xhel fragment was ligated into the BmiII/Xhel site of pK18mobSacB to yield pK18mobSacB-Tzs+Gmr. The conjugation of pK18mobSacB-Tzs+Gmr to A. tumefaciens MAF3001 rif was performed by the method of Uraji et al. (2002). After selection of the first homologous recombination with kanamycin and gentamycin, a second recombination was selected on Luria-agar medium containing Suc. Disruption of the gene was confirmed by Southern-blot analysis.

To replace tmr in the T region with tzs, TP-tzs, and AtIPT1, a 3.6-kb DNA fragment containing tmr and its upstream and downstream 1.5-kb flanking regions was amplified by PCR with pTi-SAKURA and primers Ptmr and Post-Tmr and then cloned into pCR-BluntII-TOPO to yield pTOPO-Tmrr+. For replacement with tzs and AtIPT1, SacI and AvrII sites were generated at the start and stop codons of tmr, respectively, by the QuickChange Site-Directed Mutagenesis Kit with the following primers: tmr-QC SacI and tmr-QC SacI comp for SacI, tmr-QC Avr2 and tmr-QC Avr2 comp for AvrII. The mutated plasmid was named pTOPO-Tmrr+. tzs and AtIPT1 coding regions were inserted by PCR with the following primers: Tzs SacI 5' and Tzs SacI comp for Tzs, AtIPT1 SacI 5' and IPT1-2 for AtIPT1, and then cloned into pCR-BluntII-TOPO. After confirmation of the nucleotide sequences, the SacI/SpeI fragment containing tzs or AtIPT1 was ligated into the SacI/AvrII site of pTOPO-Tmrr+. Then, SacI and AvrII-SpeI fusion sites were deleted with the QuickChange Site-Directed Mutagenesis Kit with the following primers: tmr/tzs QC5' and tmr/tzs QC3' comp for deletion of the SacI site, tmr/tzs QC3' and tmr/tzs QC3' comp for the AvrII-SpeI fusion site from tmr, tmr/ipt/tqr5' and tmr/ipt/tqr5' comp for the AvrII site, and tmr/tqr QC3' and tmr/ipt QC3' comp for the AvrII-SpeI fusion site from AtIPT1. Deletions were confirmed by nucleotide sequencing. A SpeI/SplI fragment containing tzs or AtIPT1 flanked with Tmrr upstream and downstream regions was cloned into the Xbal/SplI site of pK18mobSacB. To replace tmr with TP, the TP region (168 bp) and pK18mobSacB-TP/Tzs were amplified by PCR with the following primers: TP-IF-5' and TP-IF-3' for TP, tmr/ipt/tqr1500 IF-5' and tmr/tzs +1500 IF-3' for pK18mobSacB-TP/tzs. These fragments were ligated with the In-Fusion Dry-Down PCR Cloning Kit (Clontech). Double-crossover homologous recombinations were carried out as described above.

Tumor Formation Assays

A. tumefaciens cells were cultured overnight at 28°C in L-broth supplemented with the appropriate antibiotics. The bacterial cells were collected by centrifugation, and the cell concentrations were adjusted to 5 × 10^7 cells mL^-1 by diluting with fresh L-broth medium. The leaves of Kalanchoe plants were wounded with a toothpick. A bacterial cell suspension (2 μL, equivalent to 1 × 10^7 cells) was inoculated onto each wound area. Leaves at different developmental stages were used for inoculations. The tumors were photographed 34 d after inoculation. The tumorigenic symptoms were classified from 0 (none) to 5 (severe). The tumors were harvested by excision with a razor blade for further analysis. Rose stems were harvested by excision with a razor blade for further analysis.

RT-PCR Analysis

Eight days after Kalanchoe inoculation with A. tumefaciens total RNA was prepared from the inoculated area containing developing tumors using the Fast Pure RNA Kit (Takara) with RNase-free DNase I. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) with gene-specific primers: tmr-RT for tmr, tzs-RT for tzs, and TP-RT for TP-tzs, AtIPT1-RT for AtIPT1, and KD-265-1R for 26S rRNA. The primers used for the following PCRs were tmr RT-PCR-F1 and tmr RT-PCR-R1 for tmr, tzs RT-PCR-F3 and tzs RT-PCR-R5 for tzs, TP-RT-PCR-F4 and TP-RT-PCR-R4 for TP-tzs, AtIPT1-RT-PCR-F3 and AtIPT1-RT-PCR-R3 for AtIPT1, and KD-265-1F and KD-265-2R for 26S rRNA.

Nucleotide sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers as follows: tmr (NC_002147), tzs (NC_002147), Fd III (M7383), AtIPT1 (AB062607), and 26S rRNA (AF274651). Nucleotide sequences used in this study are as follows: Tmrr (NP_053424), Tzs (NP_053379), Fd III (BA19251), and AtIPT1 (BAB9040). The Arabidopsis Genome Initiative code for AtIPT1 is At1g68840.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Kinetic parameters of Tmr and Tzs.

Supplemental Figure S2. Effect of Met-Ala or transit peptide addition to the N terminus of Tzs on catalytic activity.

Supplemental Figure S3. Detection of transcripts in tumors on Kalanchoe by RT-PCR.

Supplemental Figure S4. Protease sensitivity assay with chloroplasts from Tzs-overexpressing Arabidopsis.

Supplemental Table S1. Quantification of cytokinins in transgenic Arabidopsis lines overexpressing A. tumefaciens IPTs.

Supplemental Table S2. Quantification of cytokinins in transgenic Arabidopsis lines overexpressing Tzs or TP-Tzs.

Supplemental Table S3. Quantification of cytokinins and auxin in galls on Kalanchoe leaves.

Supplemental Table S4. Quantification of cytokinins in galls on rose.

Supplemental Table S5. Primers used in this study.

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LITERATURE CITED


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