Oxicam-type nonsteroidal anti-inflammatory drugs enhance Agrobacterium-mediated transient transformation in plants

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Received December 1, 2021; accepted March 12, 2022 (Edited by Y. Hoshino)

Abstract Agrobacterium-mediated transformation is a key innovation for plant breeding, and routinely used in basic researches and applied biology. However, the transformation efficiency is often the limiting factor of this technique. In this study, we discovered that oxicam-type nonsteroidal anti-inflammatory drugs, including tenoxicam (TNX), increase the efficiency of Agrobacterium-mediated transient transformation. TNX treatment increased the transformation efficiency of Agrobacterium-mediated transformation of Arabidopsis thaliana mature leaves by agroinfiltration. The increase of efficiency by TNX treatment was not observed in dde2/ein2/pad4/sid2 quadruple mutant, indicating that TNX inhibits the immune system mediated by jasmonic acid, ethylene, and salicylic acid against to Agrobacterium. We also found that TNX-treatment is applicable for the transient expression and subcellular localization analysis of fluorescent-tagged proteins in Arabidopsis leaf cells. In addition, we found that TNX increases the efficiency of Agrobacterium-mediated transient transformation of Jatropha. Given that treatment with oxicam compounds is a simple and cost effective method, our findings will provide a new option to overcome limitations associated with Agrobacterium-mediated transformation of various plant species.

Key words: Agrobacterium-mediated transient transformation, Arabidopsis thaliana, Jatropha curcas, tenoxicam.

Agrobacterium-mediated transformation is a routine procedure in basic plant researches, and is a principal mean of generating transgenic plants in the agricultural and biotechnological industries (Gelvin 2005). However, heightened immune responses of plants suppress Agrobacterium-mediated transformation, and as a consequence, the efficiency of Agrobacterium-mediated transformation becomes low for some plant species. For instance, it is well established that ethylene (ET)- and salicylic acid (SA)-mediated immune responses deployed in plants is known to restrict Agrobacterium-mediated transformation (Anand et al. 2008; Gaspar et al. 2004; Lee et al. 2009; Nonaka et al. 2008a, 2008b; Yuan et al. 2007). Also, several mutants and transformants defective in immune responses have been reported to enhance the efficacy of Agrobacterium-mediated transient transformation in Arabidopsis [e.g. dde2/ein2/pad4/sid2 (Tsuda et al. 2009), GVG-AvrPto (Tsuda et al. 2012) and NahG (Rosas-Diaz et al. 2017)]. In some experimental strategies, the virulence of Agrobacterium is modified to influence the plant immunity and to increase the transformation efficiency (de Groot et al. 1998; Hiei et al. 1994; Ishida et al. 1996; Kimura et al. 2015; Komari et al. 2006; Kunik et al. 2001; Piers et al. 1996; Rashid...
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Accordingly, the control of the immune responses of target plants is considered as a key strategy to address the current inefficiencies in plant transformation by *Agrobacterium*.

During the course of previous chemical screening (Noutoshi et al. 2012), we found that oxicam-type nonsteroidal anti-inflammatory drugs (NSAIDs) (Figure 1), such as tenoxicam (TNX), function as inhibitors of SA-dependent immune responses in plants through oxidation of cytosolic redox (Ishihama et al. 2021). Here, we report that treatment with immune-inhibiting oxicams is effective in increasing the efficiency of transient transformation of Arabidopsis and Jatropha.

In *A. thaliana*, agroinfiltration of leaves has been performed using the mutants or transformants that are defective in immune responses, because triggered immune responses by *Agrobacterium* prevent transformation in Arabidopsis mature leaf cells (Rosas-Díaz et al. 2017; Sardesai et al. 2013; Tsuda et al. 2009). Meanwhile, this approach is not applicable in basic researches when investigating the functions and subcellular localizations of genes in some cases, especially when such mutations are related to the functions of the genes of interest. Based on our previous findings (Ishihama et al. 2021), we examined whether a pretreatment of *Agrobacterium* suspension with TNX would enable us to utilize wild-type Arabidopsis for agroinfiltration in a manner similar to immunity-deficient mutants.

To assess the feasibility of this approach, we added TNX (dissolved in DMSO; T0909 Sigma-Aldrich; a final concentration of 100 µM in the resuspension medium) to a suspension culture of 35S::GUS-carrying *Agrobacterium* (*Agrobacterium tumefaciens* strain Agl1 carrying the GUS reporter gene in a pGreen0029 vector) in prior to infiltration (as previously described (Choi et al. 2013) and detailed procedure in Supplementary Materials and Methods). GUS activity was detected by histochemical staining and quantitative MUG assays as an indication for the transformation efficiency (Supplementary Materials and Methods). We observed that *Agrobacterium* suspension containing TNX markedly enhanced the GUS activity in wild-type leaves by about 7-folds compared to the suspension without TNX (Figure 2A). Next, we compared if the effect of TNX is comparable to that of dde2/ein2/pad4/sid2 mutation. The quadruple mutant (dde2/ein2/pad4/sid2) is defective in ET-, SA-, and jasmonic acid (JA)-mediated immunity, thereby displaying high transformation efficiency to *Agrobacterium* by leaf infiltration (Tsuda et al. 2009). As a result, the increased GUS activity in wild-type leaves caused by TNX treatment was equivalent to that when the mutant leaves were infiltrated with *Agrobacterium* suspension without TNX (Figure 2A).

These data suggest that T-DNA is transformed more efficiently via *Agrobacterium* as the infection frequency was increased by reducing plant immunity by the TNX treatment. Subsequently, we examined the subcellular localization of expressed marker proteins, including nuclear-localized histone 2B (H2B-GFP), the trans-Golgi network (TGN) marker (Venus-SYP61), and plasma membrane-bound aquaporin (PIP2a-mCherry) following infiltration with *Agrobacterium* (*A. tumefaciens* strain GV3101 harboring RPS::H2B-GFP, 35S::Venus-SYP61, and 35S::PIP2A-mCherry) suspension containing TNX. The markers were found at the expected subcellular locations in both TNX-treated and non-treated leaf cells, when the epidermal cells were observed by a Zeiss LSM 700 inverted confocal microscope (GFP and Venus were excited at 488 nm, and emission was recorded between 501 and 545 nm, whereas mRFP was excited at 561 nm and the emission was recorded between 570 and 615 nm, Figure 2B). This indicates that TNX does not affect the subcellular localization of organelle marker proteins, and also implying that agroinfiltration of Arabidopsis

Figure 1. The structures of oxicam compounds. Tenoxicam (TNX), meloxicam (MLX), piroxicam (PRX), ampiroxicam (APRX), sudoxicam (SDX), and lornoxicam (LNX).
leaves using TNX is applicable as an efficient transient expression system to monitor the subcellular dynamics of proteins. In contrast, however, TNX treatment appeared to have no effects on stable transformation of root or flower (Supplementary Tables S1, S2).

Which immune-related pathway is targeted by TNX in recipient plants? To address this issue, we subjected the dde2/ein2/pad4/sid2 quadruple mutant to transformation using Agrobacterium suspension containing TNX. We found that the transformation efficiency, as indicated by GUS activity, was comparable to that in the mutant infiltrated with mock-treated Agrobacterium strain carrying the constructs RPS::H2B-GFP for the nucleus (big green dots), 35S::Venus-SYP61 for the trans-Golgi network (small green dots indicated by arrow heads in cropped figures), and 35S::PIP2A-mCherry for the plasma membrane (red) in the mock- or TNX (100 µM)-treated wild-type plants. Fluorescence signals in epidermal cells were observed 2 days after infiltration. Bars, 100 µm (Bars, 10 µm in cropped figures). (C and D) Quantitative MUG assay to assess the effect of TNX on quadruple mutant plants (C) and the effect of oxicams on wild-type plants (D). Agroinfiltration conditions were the same as those in (A). Different letters indicate statistically significant differences, as determined using Tukey's test (p<0.05).

Based on our finding using the vegetative tissues of A. thaliana (Figure 2), we expected that TNX treatment might also enhance the transformation efficiency of other plants that use vegetative tissues for transformation. To verify this possibility, we evaluated the effect of TNX on Agrobacterium-mediated transformation of Jatropha (Jatropha curcas L.). We chose Jatropha because the transformation methods typically utilize vegetative tissues such as cotyledon explants (Figure 3A, B). We transformed Jatropha using A. tumefaciens (strain EHA101) harboring selection vectors (PalSelect A-3 vector, Kumiai Chemical Industry Co., Ltd.) by the transformation procedure used has been described previously by Enoki et al. 2017 and Chacuttayapong et al. 2021, and added 100 µM TNX to the co-cultivation suspension. To determine the efficiency of transformation, we introduced selection vectors that enabled us to evaluate the transformation rate by PCR-based genotyping and RT-PCR for the expression of the transgenes (detailed information in Supplementary Materials and Methods; Figure 3C). We found that the transformation rate of soil-acclimated

Figure 2. Oxicam treatment enhances the efficiency of Agrobacterium-mediated transient transformation in Arabidopsis thaliana. (A and B) Leaves of wild-type and dde2/ein2/pad4/sid2 quadruple mutant Arabidopsis thaliana were mock- and tenoxicam (TNX [100 µM])-treated and inoculated with Agrobacterium carrying a 35S::GUS. (A) After 2 days, GUS enzyme activity was determined by histochemical staining and a quantitative MUG assay. The blue colour indicates GUS activity. Experiments were repeated five times with similar results. Bars represent the means and standard errors of data obtained from three biological replicates. (B) Transgenic leaves inoculated with Agrobacterium strains carrying the constructs RPS::H2B-GFP for the nucleus (big green dots), 35S::Venus-SYP61 for the trans-Golgi network (small green dots indicated by arrow heads in cropped figures), and 35S::PIP2A-mCherry for the plasma membrane (red) in the mock- or TNX (100 µM)-treated wild-type plants. Fluorescence signals in epidermal cells were observed 2 days after infiltration. Bars, 100 µm (Bars, 10 µm in cropped figures). (C and D) Quantitative MUG assay to assess the effect of TNX on quadruple mutant plants (C) and the effect of oxicams on wild-type plants (D). Agroinfiltration conditions were the same as those in (A). Different letters indicate statistically significant differences, as determined using Tukey's test (p<0.05).
plants was approximately 8-fold higher when TNX-treated suspension was used (15.14±3.61%), compared to the control (1.89±0.74%, Figure 3C). Similarly, when we calculated the transformation rate with respect to the number of cotyledon explants as starting materials, the rate was also increased by 8-folds by TNX treatment (0.285%, 3 transformants obtained from 2,954 cotyledon explants) compared to the control (0.033%, 1 transformants obtained from 1,052 cotyledon explants). Although we need further experiments to assess the effect of TNX on the stable transformation, these data suggest that pre-treatment of co-cultivation suspension with TNX can significantly enhance the efficiency of Agrobacterium-mediated transient transformation of Jatropha.

We also evaluated the effect of TNX treatment on transforming other species, namely maize, rice, soybean, Brassica napus, Brassica rapa, and water starwort. In maize (Supplementary Figure S1A), we observed that callus derived from TNX-treated immature embryos showed significantly higher survival rate (28.03±3.39%) than mock-treated control (21.67±2.97%, Supplementary Figure S1B). Furthermore, we also observed that the average size of TNX-treated callus was significantly larger (24.90±2.54 mm²) than that of mock-treated control (18.14±1.43 mm², Supplementary Figure S1C, D). The TNX treatment seems to influence on callus proliferation, though we need further assessments. Unfortunately, we failed to detect a significant effect of TNX treatment on the transformation efficiency for rice, soybean, Brassica napus, Brassica rapa, and water starwort (Supplementary Figure S2, Supplementary Tables S3–S6). However, except for water starwort, we used meristematic tissues of these plants, such as callus, callus-induced tissues, or cotyledonary nodes, rather than mature vegetative tissues for transformation, therefore, it is possible that the effect of TNX may have tissue-specificity, in addition to the species-specificity. It is well established that plant growth and immunity show antagonistic interactions; for example, young tissues must suppress the immune response to maximize growth in the absence of perceived pathogens, whereas mature organs are more adapted for defensive roles (Kadota et al. 2004). SA has been established to be a key regulator promoting immunity, but it also suppresses growth. Meanwhile, the atypical E2F protein, DEL1, promotes cell proliferation, and suppresses expression of the SA transporter gene, enhanced disease susceptibility 5 (EDS5), and the SA biosynthetic gene, ISOCHORISMAATE SYNTHASE1 (ICS1), to suppress SA accumulation and defense responses in growing tissues (Chandran et al. 2014). EDS5 and ICS1 are highly expressed in the mature tissues of A. thaliana, such as leaves, and their expressions are kept low in meristematic tissues (Supplementary Figure S3). Therefore, the spatiotemporal expression of these genes would explain why TNX was effective for leaf agroinfiltration but ineffective for transforming flowers and roots (Figure 2 and Supplementary Tables S1, S2).

In this study, we demonstrated that oxicam-type NSAIDs, including tenoxicam, enhance the efficiency of Agrobacterium-mediated transient transformation in A. thaliana and Jatropha. Since the treatment with oxicam compounds is comparatively straightforward (simply adding chemicals to the Agrobacterium co-cultivation medium) and cost effective (estimated as one US dollar for one hundred transformants), we believe that our finding will potentially provide a solution for overcoming some of the current limitations associated with Agrobacterium-mediated transformation in plants.

Acknowledgements

We thank Dr. Kenichi Tsuda (Huazhong Agricultural University) for providing seeds of the dde2/ein2/pad4/sid2 quadruple mutant, Dr. Hirokazu Tsukaya (The University of Tokyo) for providing helpful advice regarding the experiments using water starwort, and Dr. Goro Horiguchi (Rikkyo University) for providing the binary vector pH 35G.

References


Figure 3. Tenoxicam (TNX) treatment enhances the efficiency of Agrobacterium-mediated stable transformation in Jatropha. (A and B) Images of mature Jatropha plants (A) and the cotyledon used for transformation (B). Bar, 50 mm. (C) Transformation rate determined by PCR genotyping and RT-PCR in the mock- or TNX (100 µM)-treated plants. Bars represent the averages and standard errors of data obtained from two independent experiments. *p<0.05 by t-test.


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