Oxicam-type nonsteroidal anti-inflammatory drugs enhance

Agrobacterium-mediated transient transformation in plants

Seung-won Choi1,2, Kie Kumaishi3, Reiko Motoshi4, Harumi Enoki4, Wiluk Chacuttayapong1, Tadashi Takamizo5, Hiroaki Saika6, Masaki Endo6, Tetsuya Yamada7, Aya Hirose7, Nobuya Koizuka8, Seisuke Kimura9,10, Yaichi Kawakatsu9, Hiroyuki Koga11, Emi Ito2,12, Ken Shirasu1,*

Yasunori Ichihashi3,**

1Riken Center for Sustainable Resource Science, Yokohama, Kanagawa 230-0045, Japan; 2Department of Natural Sciences, International Christian University (ICU), Mitaka, Tokyo 181-8585, Japan; 3Riken BioResource Research Center, Tsukuba, Ibaraki 305-0074, Japan; 4Faculty of Agriculture, Department of Applied Life Sciences, Shizuoka University, Shizuoka, Shizuoka 422-8529, Japan; 5National Institute of Livestock and Grassland Science, Nasu-Shiobara, Tochigi 329-2793, Japan; 6Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO), Tsukuba, Ibaraki 305-8634, Japan; 7Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan; 8College of Agriculture, Tamagawa University, Machida, Tokyo 194-8610, Japan; 9Department of Industrial Life Sciences, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Kyoto 603-8555, Japan; 10Center for Plant Sciences, Kyoto Sangyo University, Kyoto, Kyoto 603-8555, Japan; 11Department of Biological Sciences, Graduate school of Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan; 12Graduate School of Humanities and Sciences, Ochanomizu University, Bunkyo-ku, Tokyo 112-8610, Japan

*E-mail: ken.shirasu@riken.jp Tel: +81-45-503-9444
**E-mail: yasunori.ichihashi@riken.jp Tel: +81-29-836-9118

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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-Díaz 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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et al. 1996; Rohini and Rao 2000; Wenck et al. 1999). 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 (TXN), 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 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 TXN 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 TXN (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 Ag11 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 TXN markedly enhanced the GUS activity in wild-type leaves by about 7-folds compared to the suspension without TXN (Figure 2A). Next, we compared if the effect of TXN 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 TXN treatment was equivalent to that when the mutant leaves were infiltrated with Agrobacterium suspension without TXN (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 TXN 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 TXN. The markers were found at the expected subcellular locations in both TXN-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 TXN does not affect the subcellular localization of organelle marker proteins, and also implying that agroinfiltration of Arabidopsis...
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 (Figure 2C). These observations thus indicate that the signaling pathways mediated by SA, JA, and ET is effectively inhibited by TNX.

We also examined the effects of five other oxicams, namely, meloxicam (MLX; dissolved in DMSO; M3935 Sigma-Aldrich; a final concentration of 100 µM in the resuspension medium), piroxicam (PRX; P5654 Sigma-Aldrich), ampiroxicam (APRX; SML1475 Sigma-Aldrich), sudoxicam (SDX; S688950 Wako Pure Chemical Industries, Ltd.), and lornoxicam (LNX; SML0338 Sigma-Aldrich; Figure 1), on the efficiency of Agrobacterium-mediated transient transformation of Arabidopsis leaves. We found that LNX and SDX were also effective, and their effect on the transformation efficiency monitored by GUS activity was comparable to that of TNX (Figure 2D). Taken together, our results suggest that oxicam treatment can be used as a new approach to enhance the efficiency of agroinfiltration to Arabidopsis leaves.

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
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, ISOCHORISMATE 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.

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