Taro (Colocasia esculenta) Transformed with a Wheat Oxalate Oxidase Gene for Improved Resistance to Taro Pathogen Phytophthora colocasiae

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Additional index words. Agrobacterium tumefaciens, genetic engineering, transgenic, disease resistance, Taro leaf blight

Abstract. Production of taro [Colocasia esculenta (L.) Schott], a tropical root crop, is declining in many areas of the world as a result of the spread of diseases such as Taro leaf blight (TLB). Taro cv. Bun Long was transformed through Agrobacterium tumefaciens with the oxalate oxidase (OxO) gene gf2.8 from wheat (Triticum aestivum). Insertion of this gene was confirmed by polymerase chain reaction (PCR) and Southern blot analysis. One independent transformed line contained one gene insertion (g5), whereas a second independent line contained four copies of the gene. Reverse transcriptase PCR (RT-PCR) confirmed the expression of this gene in line g5. Histological analysis of the enzyme oxalate oxidase confirmed its activity increased in the leaves of line g5. A bioassay for resistance to TLB used zoospores of Phytophthora colocasiae to inoculate tissue-cultured plantlets. Transgenic line g5 showed the complete arrest of this disease; in contrast, the pathogen killed non-transformed plants by 12 days after inoculation. A second bioassay, in which spores of P. colocasiae were inoculated onto disks of leaves of one-year-old potted plants, confirmed that transgenic line g5 had greatly increased resistance to this pathogen. This is the first report to demonstrate that genetic transformation of a crop species with an OxO gene could confer increased resistance to a pathogen (P. colocasiae) that does not secrete oxalic acid (OA).

Received for publication 4 Oct. 2012. Accepted for publication 7 Dec. 2012.

This project was supported by grants from the USDA-CSREES Tropical and Subtropical Agriculture Research (T-STAR) program.

The mention of a trademark, proprietary product, method, or vendor does not imply endorsement by the University of Hawaii, Hawaii Agriculture Research Center, China Academy of Tropical Agricultural Sciences, or the University of Miami nor its approval to the exclusion of other suitable products or vendors. We thank Dr. Francois Bernier at I.B.M.P. (Institut de Biologie Moléculaire Des Plantes) in France for providing the plasmid pEMBL18:gf2.8.

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Taro is a tropical root crop that is grown widely throughout the Pacific, Africa, Asia, the West Indies, and South America (Plucknett et al., 1970). It was the fifth most harvested root crop in the world with production estimated at 9.0 billion kg (Food and Agriculture Organization of the United Nations, 2010). Traditionally, taro is propagated vegetatively from corms or cormels and not from seed as a result of infrequent seed production in commercial cultivars.

Taro corms and cormels are good sources of carbohydrates, and leaves can be eaten as vegetables. They are known to contain abundant levels of both soluble oxalates and insoluble calcium oxalates (Sakai et al., 1984; Seff-Dedeh et al., 2004). Oxalate is known to function as an antinutrient in insects, animals, and humans, partly as a result of its ability to form complexes with calcium or magnesium, making these minerals unavailable for absorption (Bohn et al., 2004; Franceschi and Nakata, 2005; Palgi et al., 2005). In addition, insoluble calcium oxalate crystals could serve as herbivore feeding deterrents (Korth et al., 2006; Sakai et al., 1984). To reduce its toxicity, taro corms and leaves are well cooked before consumption by humans.

TLB, caused by the oomycete pathogen Phytophthora colocasiae, is a major disease that threatens the sustainability of taro worldwide. It reached the Hawaiian Islands during the 1920s and causes yield losses of up to 50% (Trujillo, 1967). During the 1990s, this disease spread to the Samoan Islands and resulted in 95% losses in traditional, TLB-susceptible taro cultivars (Brooks, 2000; Trujillo and Menezes, 1995). In 2004, it invaded the Dominican Republic, infecting 70% to 95% of commercial taro plantings (J. Cho, personal communications, 2009).

OxO was first isolated and characterized from wheat (Triticum aestivum) (Lane et al., 1993). It catalyzes the oxidation of oxalic acid by molecular oxygen to form carbon dioxide and hydrogen peroxide (H2O2). Researchers have found that increased OxO activity is associated with plant defense systems against a broad range of pathogens including viruses, bacteria, fungi, oomycetes, and nematodes (Dunwell et al., 2008). For example, Schweizer et al. (1999) found that transient expression of the pathogen-induced wheat germ g2f2.8 gene was associated with reduced penetration of the fungus Blumeria graminis.

There are several hypotheses to explain the mechanism of OxO in fungal resistance (Lane, 2002). First, H2O2 is generated by OxO-mediated breakdown of oxalate, resulting in hypersensitive cell death and antimicrobial activity (Peng and Kuc, 1992). Second, H2O2-mediated lignification of cell walls forms effective barriers against fungal penetration (Schweizer et al., 1999). Third, H2O2 generated by OxO could have a role in signal transduction cascades that coordinate various defense responses such as the synthesis of pathogenesis-related (PR) proteins and phytoalexins (Hammond-Kosack and Jones, 1996). Fourth, OA-generating fungi such as Sclerotinia sclerotiorum secrete high concentrations of OA as a toxin, and OxO is able to break down OA (Donaldson et al., 2001; Liang et al., 2001).

Genetic transformation with OxO genes has been reported in several plant species to improve resistance to pathogenic fungi that secrete OA (Donaldson et al., 2001; Dong et al., 2008). For example, Cober et al. (2003) found that a transgenic soybean line with an inserted wheat OxO gene gf2.8 exhibited partial resistance to the white mold disease caused by S. sclerotiorum. Peanut (Arachis hypogaea) transformed with a barley oxalate oxidase gene showed enhanced resistance to Sclerotinia minor, the pathogen that causes Sclerotinia blight of peanut (Livingstone et al., 2005). Hybrid poplar (Populus × euramericana) transformed with a wheat OxO gene exhibited enhanced disease resistance against the fungus Septoria musiva (Liang et al., 2001). Because OA is a toxin secreted by these pathogenic
Phytophthora colocasiae. This is the first report of a plant species transformed with an OxO gene that exhibited greater resistance against an oomycete pathogen, P. colocasiae, that does not secrete OA.

Materials and Methods

Plant materials. Taro cv. Bun Long was chosen as the plant transformation material, because it is an important TLB-susceptible commercial cultivar in Hawaii. Earlier, we had developed an efficient regeneration and Agrobacterium-mediated transformation system for this cultivar (He, 2006; He et al., 2008).

Agrobacterium-mediated transformation. The plasmid pEMBL18:gf2.8 was provided by Dr. Francois Bernier at I.B.M.P. (Institut de Biologie Moleculaire des Plants) in France. Enzyme digestion followed by PCR analysis and partial sequencing verified that this plasmid contained the intact 2.8-kb gf2.8 gene including its own promoter and terminator. The promoter for this gene is stimulated by both abiotic stresses (e.g., heavy metals, wounding, and plant growth regulators) and by biotic stresses (e.g., virus, fungus) (Berna and Bernier, 1999).

The intact 2.8 kb OxO gf2.8 gene fragment was extracted from the plasmid pEMBL18:gf2.8 with the enzyme EcoRI and ligated into the EcoRI site of the vector pH121 using standard methods (Sambrook and Russell, 2001). The plasmid pH121:gf2.8 contained the nptII selection gene, the gus reporter gene, and the intact gf2.8 gene with promoter and terminator (Fig. 1). The plasmid pH121:gf2.8 was transformed into the Agrobacterium tumefaciens strain EHA105 using the freezing and thawing method described earlier by He et al. (2008). Briefly, leaves and shoots were sliced and immersed in 10 mg L⁻¹ 1-naphthaleineacetic acid (NAA) solution for 2 h to induce gene expression because the promoter of gf2.8 is induced by this hormone. Then, sliced leaves and shoots were placed in a solution containing oxalic acid (2.5 mM) and 4-chloro-1-naphthol (0.6 g L⁻¹) as the staining reagent. The incubation was conducted at 25 °C in the dark for 24 h. The OxO converts oxalic acid to H₂O₂ that endogenous peroxidases use to oxidize 4-chloro-1-naphthol, producing a dark blue precipitate (Dumas et al., 1995).

Bioassay of transgenic plants challenged by Phytophthora colocasiae. Six-month-old transgenic plantlets were propagated from one-year-old potted plants, modified by the method of Brooks (2000). The youngest fully expanded mature leaves were selected from potted plants of one-year-old transgenic line g5 or non-transformed ‘Bun Long’ control plants. Leaf-disk (20 mm in diameter) were excised with a cork borer. Leaf-disks were immediately placed in water agar plates. Twenty microliters of spore suspension (1 × 10⁸ spores/mL) were pipetted onto the center of each leaf-disk, and the plates were placed in a growth chamber maintained at 24 °C, 12 h light, and 100% relative humidity. Leaf-disks were observed daily and necrotic lesions were measured at 3 d after inoculation. Six leaf-disks from three individual plants of the g5 line and six leaf-disks from three individual plants of the non-transformed control were inoculated with the pathogen. Results of each leaf disk were averaged per treatment. Each experiment was repeated three times and data analyzed statistically by ANOVA using the GLM program of SAS software (Statistical Analysis System).

To compare the TLB resistance of the transgenic line g5 to naturally occurring TLB-susceptible and TLB-resistant cultivars, a third leaf-disk bioassay was performed using the Hawaiian commercial cultivar Maui Lehua (susceptible to TLB), the transgenic line g5, and three putative TLB-resistant cultivars, Pa’akala, BC99-6, and Ngesuas (P1). Cultivar Ngesuas was introduced from Palau based on observed field resistance to TLB; cv. Pa’akala was bred conventionally by Trujillo for increased TLB resistance (Trujillo et al., 2002); and cv. BC99-6 was bred conventionally by Cho for greater TLB resistance Cho et al. (2007). The bioassay was conducted as described earlier, except for placing five leaf-disks on each plate.

Plantlets were inoculated with the oomycete pathogen P. colocasiae according to He et al. (2008). Briefly, a plug of V8 agar (Miller, 1955) bearing active spores (≈0.2 cm²) was placed spore side down on the cut shoot base of each plantlet. Each inoculated plantlet was placed on moistened filter paper and sealed in a petri dish to maintain 100% humidity. Three non-transformed plantlets and three transgenic plantlets of the line g5 were used in each trial, and each trial was repeated three times using a randomized complete block design. After inoculation, plantlets were observed daily for lesion initiation for 30 d. At 12 d, the lesion diameters were measured and averaged across three plantlets, and analyzed statistically by analysis of variance (ANOVA). The general linear model (GLM) program of SAS software (SAS Institute, Inc., Cary, NC) was used to conduct the ANOVA.

A second leaf-disk bioassay was performed with one-year-old potted plants, modifying the method of Brooks (2000). The youngest fully expanded mature leaves were selected from potted plants of one-year-old transgenic line g5 or non-transformed ‘Bun Long’ control plants. Leaf-disk (20 mm in diameter) were excised with a cork borer. Leaf-disks were immediately placed in water agar plates. Twenty microliters of spore suspension (1 × 10⁸ spores/mL) were pipetted onto the center of each leaf-disk, and the plates were placed in a growth chamber maintained at 24 °C, 12 h light, and 100% relative humidity. Leaf-disks were observed daily and necrotic lesions were measured at 3 d after inoculation. Six leaf-disks from three individual plants of the g5 line and six leaf-disks from three individual plants of the non-transformed control were inoculated with the pathogen. Results of each leaf disk were averaged per treatment. Each experiment was repeated three times and data analyzed statistically by ANOVA using the GLM program of SAS software (Statistical Analysis System).

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survived after 90 d of selection on 50 mg L⁻¹ G418 and were transferred to the shoot-inducing medium containing the 50 mg L⁻¹ G418 with subculturing every 30 d. After 90 d, 30 independent shoot lines were initiated on the selection media. After another 60 d, only 10 lines survived the selection. PCR analysis was performed to screen transgenic lines. The expected 755-bp PCR product specific for the gf2.8 gene fragment was found in eight lines (Fig. 2A), indicating that the gf2.8 gene had been successfully transformed into these lines. No PCR product was obtained from the DNA extracted from non-transformed tao.

In addition, RNA was isolated from these eight lines and RT-PCR analysis conducted. The expected 755-bp RT product specific for the gf2.8 gene fragment was found in all eight lines (Fig. 2B), indicating expression of the inserted gene. There was no RT product specific for the gf2.8 gene fragment from RNA of non-transformed tao.

Restriction digests of genomic DNA extracted from the eight transgenic lines, using the enzyme BamHI, yielded various bands (Fig. 2C). Transgenic lines 1 to 6 (g1–g6) appeared to have the same pattern, indicating one independent transformation event. Transgenic lines g7 and g8 appeared to have the same integration pattern, indicating a second independent transformation event. A single band was found in g1–g6 and four bands were found in g7–g8 (Fig. 2C), indicating a single site insertion and a multiple (i.e., four) site insertion, respectively, because BamHI only has one digestion site in the T-DNA. This result confirmed the presence and intactness of the transgene gf2.8 in the transformed tao genome. In the histochemical analysis of OxO activity, a dark blue precipitate was found in leaves and shoots of the NAA-induced transformed g5 taro line but not in the non-transformed control (Fig. 2D). This result confirmed the increased level of this protein in transgenic line g5.

In a bioassay of one-year-old plants, inoculation of leaf disks from non-transformed tao with zoospores of P. colocasiae resulted in chlorotic and necrotic, water-soaked lesions within 24 h after inoculation. This lesion area increased over time, resulting in necrosis of the whole leaf disk of control plants within 3 d after inoculation (Fig. 3C, left). In contrast, leaf disks of transgenic line g5 did not exhibit water-soaked lesions within 24 h after inoculation, and only small lesions occurred at the inoculation area within 3 d after inoculation (Fig. 3C, right). The average lesion diameters measured at 3 d after inoculation were significantly smaller (P < 0.05) in transgenic plantlets of line g5 with the wheat oxalate oxidase gene gf2.8 compared with those of the non-transformed plantlets (Fig. 3B).

In the third bioassay, necrotic lesions were observed on all leaf disks of susceptible cultivar Maui Lehua within 24 h after inoculation. This lesion area increased over time, resulting in necrosis of the whole leaf disk of cv. Maui Lehua within 3 d after inoculation (Figs. 3E, left). In contrast, much smaller lesions were evident on several leaf disks of TLB-resistant cultivar Pa‘akāla (Figs. 3E, middle) and all leaf disks of transgenic line g5 (Figs. 3E, right) within 24 h after inoculation. The white mycelium of P. colocasiae could be observed on several leaf disk surfaces of line g5, but there were either no lesions or only a few lesions of limited diameter that developed (Fig. 3E, right). Both cv. Pa‘akāla and transgenic line g5 showed significantly smaller lesions than cv. Maui Lehua, BC99-6, and P1 at 3 d after inoculation (Fig. 3E–F). There was no significant difference in average lesion diameter between transgenic line g5 and cv. Pa‘akāla (Figs. 3F).

**Discussion**

Transformation of tao with the OxO gene gf2.8 from wheat showed great promise in controlling the major tao pathogen *P. colocasiae*, particularly in tao cultivars...
Fig. 3. Continued.
that do not lend themselves to conventional cross-breeding. The spread of this oomycete pathogen was completely stopped in transgenic plantlets and greatly inhibited in leaf blades of one-year-old cv. Bun Long plants. To our knowledge, this is the first report of a transgenic plant with an inserted OxO gene that exhibited enhanced resistance to a pathogen (*P. colocasiae*) that does not secrete OA. These results are of particular interest, because Taro leaf blight caused by *P. colocasiae* is one of the worst, most invasive diseases affecting the sustainability of taro production in the world.

This study provides evidence in support of either a direct effect of H$_2$O$_2$ (e.g., hypersensitive-like cell death or antimicrobial activity) or an indirect effect of H$_2$O$_2$ (e.g., cell wall lignification or induction of PR genes) on plant responses to pathogens. We have observed hypersensitive-like responses on transgenic line g5 plantlets (Fig. 3A, right); in addition, mycelium of *P. colocasiae* appeared to grow on the surface of the leaf disk but did not penetrate into the leaf tissue (Fig. 3C, right). Interestingly, Urs et al. (2006) reported that localized H$_2$O$_2$ levels and peroxidase activity increased when gametophytes of the fern *Ceratopteris richardii* were exposed to pathogen *Pythium infestans*, indicating that in this plant species, peroxidases may be involved in production of H$_2$O$_2$ and resistance to an oomycete pathogen.

One advantage of genetic transformation is that disease resistance gene(s) can be inserted into the genome of an elite cultivar, while maintaining all of its other desirable characteristics. Cultivar Bun Long is commercially important in Hawaii and the Dominican Republic; however, it flowers rarely under the environmental conditions found in these two islands. As a result of the difficulty in the induction of flowering of cv. Bun Long (even with the use of gibberellic acid that is effective on other taro cultivars), conventional breeding of this particular cultivar for increased disease resistance is extremely slow. In contrast, commercially important cv. Maui Lehua is more amenable to conventional breeding, because it flowers naturally in Hawaii and in response to gibberellic acid. Although cv. Maui Lehua showed significantly less resistance to TLB than the transgenic line g5, its progeny, Pa’akala, exhibited similar resistance to the transgenic line g5. These results indicate that conventional breeding for increased TLB resistance is possible...
for elite cultivars that flower more readily such as cv. Maui Lehua.

Sharma et al. (2009) did not report up-regulation of OXO genes in resistant taro cv. Muktakeshi compared with susceptible taro cv. UL-56 when each was infected by *P. colocasiae*. This result indicates that other PR proteins (e.g., chitinases) may be involved in TLB resistance of that particular taro cultivar. Interestingly, in previous research, taro transformed with the chitinase gene RICCH11 showed no change in resistance to the oomycete pathogen *P. colocasiae* (He et al., 2008, 2010).

Another advantage of genetic transformation to increase disease resistance is the ability to stack genes of interest (Sridive et al., 2008). In previous research, transgenic taro with an inserted chitinase gene from rice showed moderately increased tolerance of the fungal pathogen *Sclerotium rolfsii* (He et al., 2008, 2010). Insertion of the OXO gene into taro also resulted in a modest increase in resistance to *S. rolfsii* (He, 2006). Perhaps, disease resistance could be enhanced further in future transgenic taro plants by the combined expression of a chitinase gene with the OXO gene as found in other plant species.

Despite the potential advantages of genetic transformation of taro with the OXO gene to confer enhanced disease resistance, such research in Hawaii has been suspended as a result of controversies discussed briefly by He et al. (2010). Research in our laboratory now focuses on identification of naturally occurring disease resistance genes in taro and marker-assisted selection to accelerate introgression of such genes into elite germplasm.

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