Arch. Biol. Sci., Belgrade, 67(3), 957-964, 2015 DOI:10.2298/ABS141201058C

PHENOTYPIC PERFORMANCE OF TRANSGENIC POTATO (*SOLANUM TUBEROSUM* L.) PLANTS WITH PYRAMIDED RICE CYSTATIN GENES (*OCI* AND *OCII*)

Aleksandar Cingel[1,*], Jelena Savić[1], Tatjana Ćosić[1], Martin Raspor[1], Nabil Ghalawenji[1], Ann Smigocki[2] and Slavica Ninković[1]

[1] *Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade*, Belgrade, Serbia

[2] *Molecular Plant Pathology Laboratory, USDA-ARS*, Beltsville, MD 20705, USA

[*] **Corresponding author:** cingel@ibiss.bg.ac.rs

Abstract: The evaluation of transgenic plants which is usually carried out under controlled conditions in culture rooms and greenhouses can yield valuable information about the influence of introduced genes on a transgenic plant phenotype. However, an overall assessment of plant performance can only be made by testing transgenic plants in the field environment. Thus, the effects of pyramided rice cystatin genes *OCI* and *OCII* on morphological parameters of transgenic potato cv. Desiree, Dragačevka and Jelica lines were compared under *in vitro*, greenhouse, and field conditions. All analyzed OC co-expressing transgenic lines exhibited normal phenotype, both *in vitro* and in greenhouse conditions. In the field environment, eight of nine OCI/OCII lines were similar to the wild-type control plants in their general phenotypic appearance. Yield parameters, such as tuber number and tuber weight for these phenotypically normal OCI/OCII lines, were also comparable to the controls. Only transgenic cv. Jelica line 4 plants exhibited slightly reduced growth, atypical leaf morphology and, contrary to the plants of other transgenic lines and untransformed controls, failed to flower. However, despite the phenotypic and developmental changes under field conditions, the OCI/OCII Jelica line 4 did not exhibit a significant decrease in tuber yield. Stacking of *OCI* and *OCII* genes preserves important attributes of the parental lines, confirming that this approach could be suitable for improving agronomical traits in potato.

Key words: transgenic potato plants; rice cystatins; *OCI*; *OCII*; field performances

Received December 1, 2014; **Revised** December 25, 2014; **Accepted** December 27, 2014

INTRODUCTION

Improvement of plant traits using a genetic transformation approach requires the recovery of transgenic lines with adequate heterologous gene expression while preserving the best genetic attributes of the parental line. The somaclonal variation inherent to *in vitro* plant culture or insertion mutagenesis that can occur during the plant transformation process itself can interfere with recovery of phenotypically normal transgenic plants (Conner, 2007; Barrell and Conner, 2011).

Even in the absence of genetic transformation, some clonally propagated species, such as potato, are known to be prone to tissue culture-induced somaclonal variation (Mitten et al., 1990). The frequency of somaclonal variation in various transgenic populations of potato cultivars has been reported to be between 15 and 80% (Conner et al., 1994; Meiyalaghan et al., 2011). Using sexual hybridization to eliminate somaclonal variation in potato is unfeasible due to loss of genetic integrity of the original line, while asexual reproduction permanently retains the hemizygotic status of the introduced transgenes. Thus,

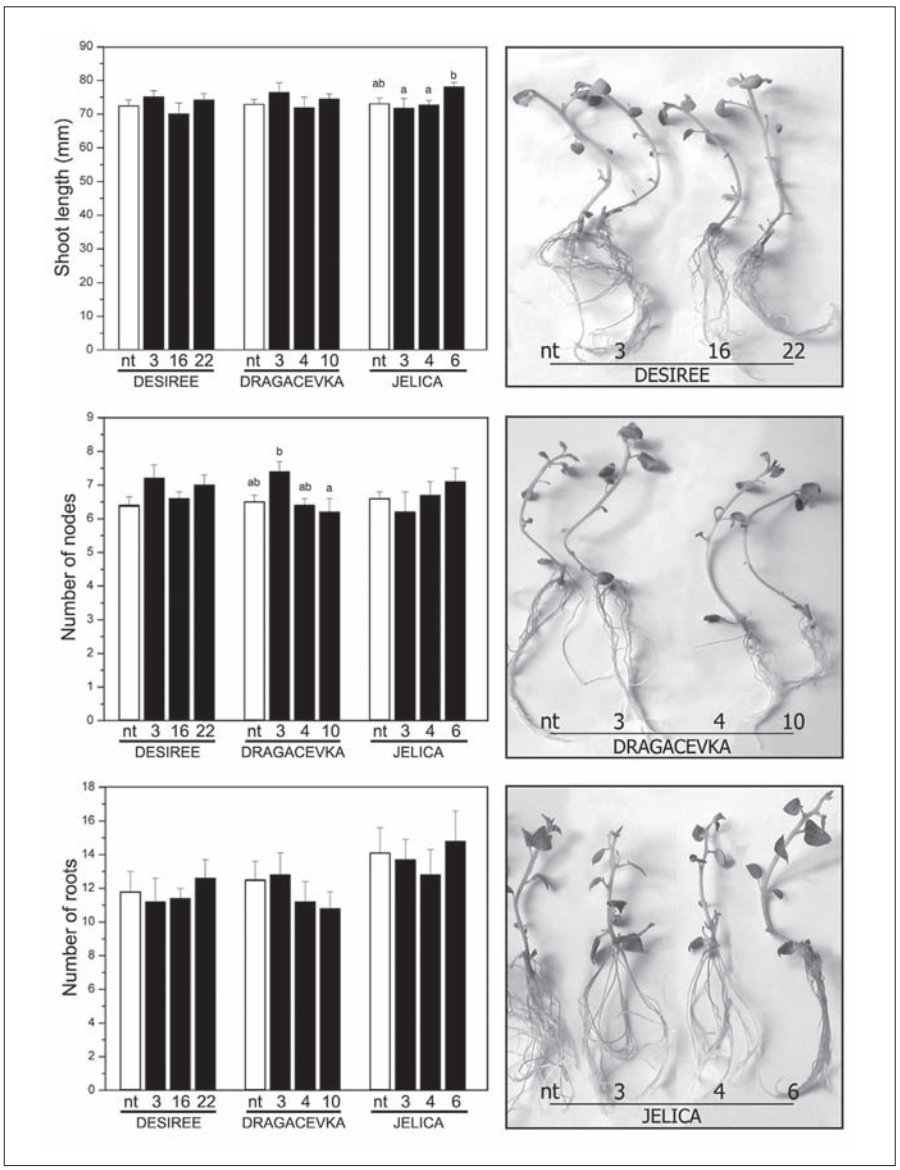

Fig.1. Morphological parameters of *in vitro*-grown Desiree lines 3, 16 and 22; Dragačevka lines 3, 4 and 10; Jelica lines 3, 4 and 6; nt – corresponding non-transformed controls. Results are expressed as mean ± SE. Means marked with different letters are significantly different according to LSD test ($P<0.05$).

potato transgenic lines are usually maintained as vegetative clones, starting with the initial selection of putative transformants all the way through to the possible eventual commercialization (Conner, 2007).

Genetic changes that lead to altered/atypical phenotypes can appear because of irreversible disruption of endogenous plant DNA after heterologous gene integration into the plant genome (Wilson et al., 2006). It was documented that for many plant species, including *Solanaceae* (Lindsey et al., 1993), T-DNA insertion frequency within coding or regulatory gene sequences was above 50%. In addition, insertion-site mutations can change expression patterns of nearby host genes, especially if the heterologous gene is under the control of a strong promoter (Ichikawa et al.,

2003). Another type of hereditary mutation connected with plant transformation are genome-wide mutations, reflected as DNA polymorphism between transgenic and wild-type plants (Sala et al., 2000). These mutations are of epigenetic nature and it is proposed that the same mechanism is involved in somaclonal variations, a phenomenon attributed to *in vitro* culture (Wu et al., 2009).

Unintended effects of transgene insertion or the transformation process itself are variable and unpredictable and, therefore, are distinct for each independently derived transgenic line. Some of these so-called pleiotropic effects can occur as a result of unexpected interference of the transgene product with plant host metabolism (Schluter et al., 2010). There are numerous examples of unintended agronomic performance changes, both negative and positive, generated by transformation and/or transgene expression (see references in Conner and Jacobs, 2000; Kabouw et al., 2012).

Although the majority of aberrant transformed plants can be discovered and removed during the early screening phase for putative transformants, there are reports that document normal *in vitro*- or greenhouse-grown transgenic lines that exhibited unexpected traits only when transferred to the more variable field environments. These unintended traits include lower yields (Casler et al., 2002; Zeller et al., 2010), altered pest or pathogen resistance (Birch et al., 2002; Munger et al., 2010) and changed plant reproductive characteristics (Bergelson et al., 1998).

In this paper, we compare the phenotypic and developmental effects of heterologous gene expression in transgenic potatoes, *Solanum tuberosum* cultivars Desiree, Dragačevka and Jelica grown *in vitro*, and under greenhouse and field conditions. All investigated transgenic potato lines express two stacked rice cystatin genes coding for cysteine proteinase inhibitors OCI and OCII that were introduced to evaluate their effect on pest resistance.

MATERIALS AND METHODS

Plant material

The transformed potato lines used in this study were derived by *Agrobacterium tumefaciens*-mediated co-transformation of Desiree and Dragačevka cultivars with the rice *OCI* and *OCII* genes (Cingel et al., 2014). Transformed Jelica cultivar lines were obtained by re-transforming of an *OCI* expressing line with the *OCII* gene (Cingel et al., 2014). Each *OCI* and *OCII* gene was fused to the wound inducible *pin2* promoter and cloned into plant transformation vectors pGV-GFP-OCI-3.8(19) or pGVGFP-OCII-4.2A7, respectively, that were maintained in a separate *Agrobacterium*

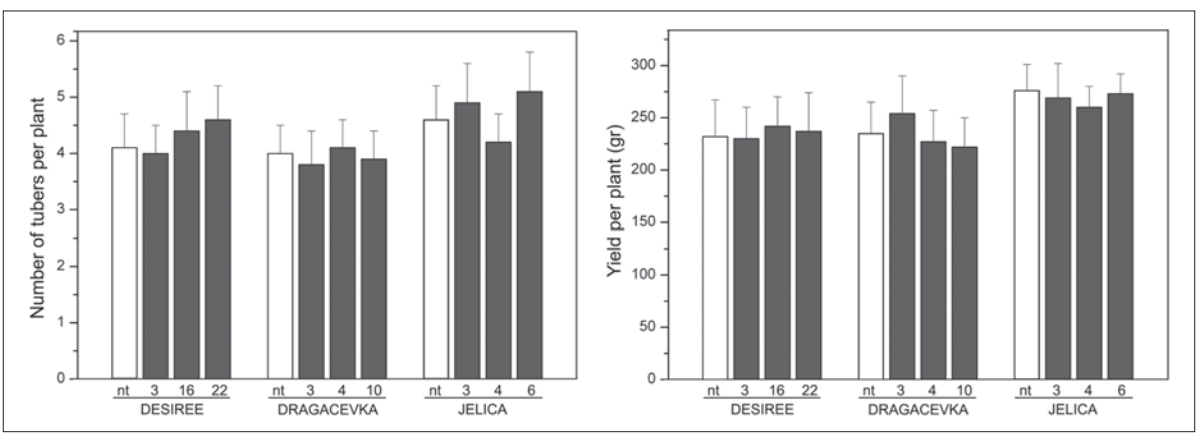

Fig. 2. Tuber production under greenhouse conditions of Desiree lines 3, 16 and 22; Dragačevka lines 3, 4 and 10; Jelica lines 3, 4 and 6; nt – corresponding non-transformed control. Results are expressed as mean ± SE. According to LSD test ($P<0.05$) there were no significant differences in the number of tubers and tuber weight per plant between individual OCI/OCII potato lines and corresponding non-transformed controls.

tumefaciens strain EHA101. Nine selected transformed *OCI*/*OCII* potato lines (Desiree lines 3, 16 and 22; Dragačevka lines 3, 4 and 10; Jelica lines 3, 4 and 6) were molecularly analyzed to confirm the wound induction of the *OCI* and *OCII* transcripts and the accumulation of biologically active OCI and OCII recombinant proteins (Cingel et al., 2014).

Growth in vitro and under greenhouse conditions

Transformed potato lines were micropropagated *in vitro* on basal MS medium containing Murashige and Skoog (1962) mineral salts, Linsmaier and Skoog (1965) vitamins, 3% sucrose and 100 mg l^{-1} myoinositol, with 0.6% agar. Lines were maintained *in vitro* in a growth room at 25±2°C and a 16 h day length. The plantlets were subcultured by transferring nodal segments to fresh medium every 5 weeks. Rooted 5-week-old plants were transferred to potting soil-vermiculite mixture (3:1) and grown under greenhouse conditions (21±4°C). After three months, developed tubers were collected in paper bags and stored in the dark at room temperature.

Growth of T1 generation in experimental field

Overwintered sprouted tubers collected from primary transformants grown in the greenhouse were planted in the experimental field. Each transgenic line and corresponding control was planted in a separate row spaced at 0.75 m. Within rows, tubers were planted in small plots that were 0.3 m apart in replicates of 20-25. Tubers were planted at the end of April 2011 and new generation (T1) tubers were harvested at the end of September 2011. During the growing period, no pesticides were applied and fields were irrigated regularly. Tubers from each plot were hand harvested and stored in paper bags.

Evaluation of phenotypic characteristics of transgenic lines

For phenotype evaluation of morphological parameters, 20 transformed and non-transformed plants of each line, in three replicates, were analyzed. After 5 weeks of *in vitro* culture, the main shoot length, number of nodes and number of roots per plant were determined. Three months after *in vitro* plantlets had been transferred to the greenhouse, survival rates, as well as parameters of tuber yield performance, i.e. number of developed tubers per plant and weight of tubers, were scored in addition to the above-listed morphological parameters.

Evaluation of field-grown T1 potato plants occurred after five months from planting the tubers originated from T0 plants. The same parameters as for greenhouse-grown plants were scored, with additional screening of developmental stages such as flowering and fruit producing.

Data analysis

For statistical analysis of quantitative morphological traits, one-way analysis of variance (ANOVA) was performed using Statgraphic Plus Version 2.1. software. Results are expressed as mean ± standard error (SE). The differences found among means were determined using Fisher's least significant difference (LSD). The acceptance level of statistical significance was $P < 0.05$.

RESULTS AND DISCUSSION

Minimizing the appearance of unintended atypical plants upon genetic transformation is vital for the improvement of agricultural performance of potato plants. This is commonly achieved by the creation of a large transgenic population at the beginning, and selection of few lines with normal phenotype and adequate transgene expression at the very end of transformation. Among a large number of regenerated plantlets, typical for potato, usually those that had undergone early regeneration events were selected (Davidson et al., 2002; Jacobs et al., 2009). This is based on the widespread assumption that a minimum duration of *in vitro* tissue culturing leads to a minimum probability of somaclonal variations. Contrary to this assumption, Barrell and Conner (2011) and Meiyalaghan et al. (2011) have demonstrated that

later potato regeneration events emerge more phenotypically normal plants than earlier ones.

During stacking rice cystatins in potato cultivars Desiree, Dragačevka and Jelica, adoption of regenerated potato shoots from induced calli was random, and healthy looking putative transformants were visually selected for further propagation and analyses, after their rooting on selective medium. This approach resulted in a phenotypically normal appearance of selected Desiree, Dragačevka and Jelica transformed lines under *in vitro* conditions (Fig. 1). Shoot length, number of nodes and roots were comparable to the non-transformed control plants (Fig. 1), indicating that the overall phenotype of the transformants was normal.

When micropropagated plants were transferred to the greenhouse, no significant difference in survival rates was noted between transformed and control plants. Survival rates for both OCI/OCII lines and wild-type potatoes were above 93%. All acclimatized transformed plants exhibited normal phenotypes with

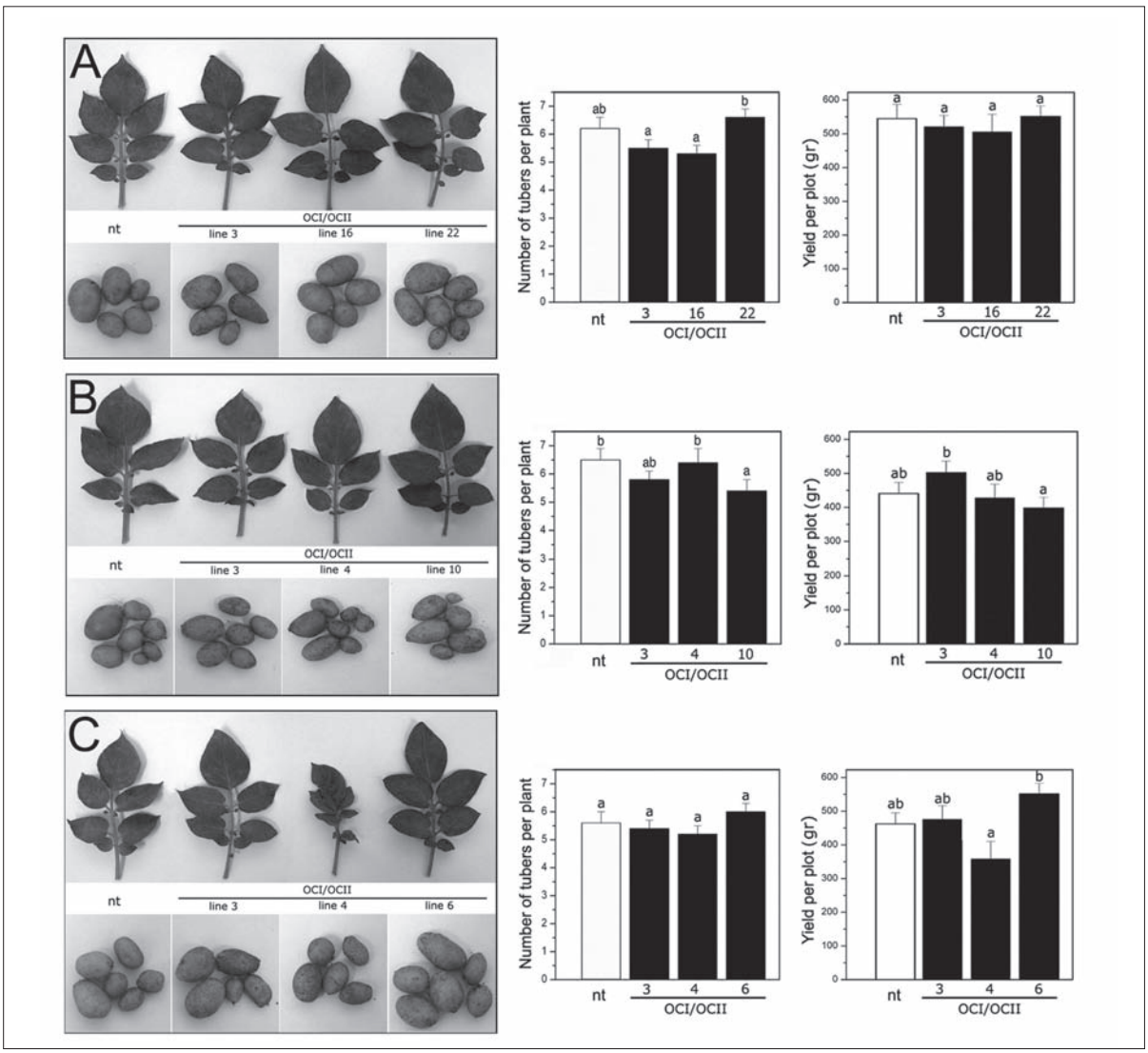

Fig. 3. Leaf morphology and tuber production in T1 progenies of non-transformed control (nt) and OCI/OCII lines grown under field conditions. (A) Desiree; (B) Dragačevka; (C) Jelica. Results are expressed as mean ± SE. Means marked with different letters are significantly different according to LSD test ($P<0.05$).

respect to elongation and leaf morphology (data not shown). Also, tuber production for the transformed lines was similar to the control plants (Fig. 2).

Additionally, assessment of the phenotypic performance of the derived OCI/OCII T1 generation under field conditions did not reveal any phenotypic variations in 8 of the 9 transgenic lines as compared to the control (Fig. 3). All regular transgenic lines flowered but did not produce fruits, like the control plants. Only Jelica transgenic line 4 plants exhibited visible phenotypic differences. These plants had slightly reduced growth, atypical leaf morphology (Fig. 3c) and failed to flower. However, despite observed alterations, OCI/OCII Jelica line 4 bore normally shaped tubers with average tuber number and weight similar to wild-type Jelica plants (Fig. 3c). Similar alterations from normal potato phenotype – decreased growth, atypical leaf morphology and absence of flowering – have been already observed for transformed field-grown potato (Conner et al., 1994). Other unintended phenotypic effects of field-grown potato after heterologous gene integration, including senescence patterns, leaf architecture, tuber weight and number, were documented also by Dale and McPartlan (1992), as well as in recent works of Richter et al. (2011) and Torres et al. (2012).

Unexpected phenotype of transgenic lines can be a direct result of heterologous gene expression, especially in the case of recombinant specific proteinase inhibitors (PI) such as the introduced oryzacystatins OCI and OCII. The potential interactions may appear due to structural homology of endogenous plant proteinases with PI target proteinases (Goutlet et al., 2008). The cysteine proteinases of higher plants are involved in numerous physiological processes (Solomon et al., 1999) and, for instance, although initial studies of heterologous *OCI* gene expression failed to find any phenotypic deviation of transgenic tobacco plants (Masoud et al., 1993), later evaluation revealed alterations in morphological parameters, such as faster growth, increase in biomass, earlier flowering (Gutierrez-Campos et al., 2001), increase of leaf protein content (Prins et al., 2008) or, contrary to this, decreased growth, smaller leaves and decrease in biomass (Van der Vyver et al., 2003). Similarly, heterologous expression of insecticidal proteins, such as lectins, agglutinins or CpTi PI in potato can decrease the number and biomass of transgenic potato leaves (Birch et al., 2002). From the other side, constitutive expression of CDI, aspartate PI (Brunelle et al., 2004) or cysteine PI, OCI and OCII (Benchekroun et al., 1995; Cingel et al., 2010) had no visible influence on transgenic potato phenotype. Also, co-expression of two different insecticidal proteins, *Bt cry* genes in potato (Meiyalaghan et al., 2010) or sporamin and CeCPI in tobacco (Senthilkumar et al., 2010) had no documented effects on transgenic plants' phenotype. Similarly, we observed no differences in OCI/OCII potato lines. The absence of direct affinity of recombinant oryzacystatins and potato proteinases (Benchekroun et al., 1995) could explain the normal development and phenotype of OCI/OCII potato lines.

On the other hand, phenotypic alteration of OCI/OCII Jelica line 4 could be connected with *in vitro* culture and regeneration phase during the plant transformation process. Phenotypical and physiological alterations were well known for plant tissue culture (Karp, 1991), and can also be frequently observed for field-growing transformed potato (Conner et al., 1994; Davidson et al., 2002; Richter et al., 2011). Our results showed, similar to other studies, that transgenic potato performance was more evident in the fluctuating field conditions of additional stress than in the more stable and controlled environment of greenhouse or *in vitro* culture, making this evaluation necessary for the selection of transformed potato lines with all parental phenotypic attributes maintained.

We conclude that the developed potato transformation method, used for stacking rice cystatin genes together with efficient selection and regeneration of transformed plants, reduces and minimizes the frequency of tissue culture-induced variation in plant performance. This can facilitate the development of transgenic lines that, along with the desired introduced improvement, retain all the elite traits of the original parent cultivars. For clonal crops, such as potato, which is highly heterozygous with diverse crossing compatibility obstacles and requirements for the screening of quite large populations of putative transformed lines, it is particularly important to recover the transgenic lines with agronomic performance similar to the wild-type cultivar.

Acknowledgements: This research was funded by Serbian Ministry of Education, Science and Technological Development (Project ON173015).

Authors' contributions: S.N. and A.S. designed research; A.C. and J.S. performed experiments and wrote the manuscript; T.Ć. and M.R. did statistical analyses; N.G. maintained plants in greenhouse and field.

Conflict of interest disclosure: Authors state that there is no conflict of interest.

REFERENCES

Barrell, P. J. and *A. J. Conner* (2011). A strategy to facilitate the recovery of phenotypically normal transgenic lines in clonal crops. *Theor. Appl. Genet.* **122**, 1171-1177.

Benchekroun, A., Michaud, D., Nguyen-Quoc, B., Overney, S., Desjardins, Y., and *S. Yelle* (1995). Synthesis of active oryzacystatin I in transgenic potato plants. *Plant Cell Rep.* **14**, 585-588.

Bergelson, J., Purrington, C. B. and *G. Wichmann* (1998). Promiscuity in transgenic plants. *Nature.* **395**(6697), 25.

Birch, A. N. E., Geoghegan, I. E., Griffiths, D. W. and *J. W. McNicol* (2002). The effect of genetic transformations for pest resistance on foliar solanidine-based glycoalkaloids of potato (*Solanum tuberosum*). *Ann. App. Biol.* **140(2)**, 143-149.

Brunelle, F., Cloutier, C. and *D. Michaud* (2004). Colorado potato beetles compensate for tomato cathepsin D inhibitor expressed in transgenic potato. *Arch. Insect Biochem.* **55**, 103-113.

Casler, M. D., Buxton, D. R. and *K. P. Vogel* (2002). Genetic modification of lignin concentration affects fitness of perennial herbaceous plants. *Theor. Appl. Genet.* **104**, 127-131.

Cingel, A., Vinterhalter, B., Vinterhalter, D., Ćalić-Dragosavac, D., Smigocki, A. and *S. Ninković* (2010). *Agrobacterium*-mediated transformation of two Serbian potato cultivars (*Solanum tuberosum* L. cv. Dragačevka and cv. Jelica). *Afr. J. Biotechnol.* **30(9)**, 4644-4650.

Cingel, A., Savić, J., Ćosić, T., Zdravković-Korać, S., Momčilović, I., Smigocki, A. and *S. Ninkovic* (2014). Pyramiding rice cystatin *OCI* and *OCII* genes in transgenic potato (*Solanum tuberosum* L.) for resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say). *Euphytica.* **198**(3), 425-438.

Conner, A. J., Williams, M. K., Abernethy, D. J., Fletcher, P. J. and *R. A. Genet* (1994). Field performance of transgenic potatoes. *NZJ Crop Hortic. Sci.* **22**, 361-371.

Conner, A. J. and *J. M. E. Jacobs* (2000). Food risks from transgenic crops in perspective. *Nutrition.* **16**, 709-711.

Conner A. J. (2007). Field-testing of transgenic potatoes. In: *Potato Biology and Biotechnology: Advances and Perspectives*. (Ed Vreugdenhil, D), 687-703. Elsevier, Amsterdam.

Dale, P. J. and *C. McPartlan* (1992). Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings. *Theor. Appl. Genet.* **84**, 585-591.

Davidson, M. M., Jacobs, J. M. E., Reader, J. K., Butler, R. C., Frater, C. M., Markwick, N. P., Wratten, S. D. and *A. J. Conner* (2002). Development and evaluation of potatoes transgenic for a cry1Ac9 gene conferring resistance to potato tuber moth. *J. Am. Soc. Hort. Sci.* **127**, 590-596.

Goulet, M. C., Dallaire, C., Vaillancourt, L. P., Khalf, M., Badri, M. A., Preradov, A., Duceppe, M. O., Goulet, C., Cloutier, C. and *D. Michaud* (2008). Tailoring the specificity of a plant cystatin toward herbivorous insect digestive cysteine proteases by single mutations at positively selected amino acid sites. *Plant Physiol.* **146**, 1010-1019.

Gutierrez-Campos, R., Torres-Acosta, J. A., Perez-Martinez, J. D. J. and *M. A. Gomez-Lim* (2001). Pleiotropic effects in transgenic tobacco plants expressing oryzacystatin I gene. *HortSci.* **36**, 118-119.

Ichikawa, T., Nakazawa, M., Muto, S., Gohda, K., Suzuki, K., Ishikawa, A., Kobayashi, H., Yoshizumi, T., Tsumoto, Y., Tsuhara, Y., Lizumi, H., Goto, Y. and *M. Matsui* (2003). Sequence database of 1172 T-DNA insertion sites in *Arabidopsis* activation-tagging lines that showed phenotypes in T1 generation. *Plant J.* **36**, 421-429.

Jacobs, J. M. E., Takla, M. F. G., Docherty, L. C., Frater, C. M., Markwick, N. P., Meiyalaghan, S. and *A. J. Conner* (2009). Potato transformation with modified nucleotide sequences of the cry9Aa2 gene improves resistance to potato tuber moth. *Potato Res.* **52**, 367-378.

Kabouw, P., van Dam, N. M., van der Putten, W. H. and *A. Biere* (2012). How genetic modification of roots affects rhizosphere processes and plant performance. *J. Exp. Bot.* **63**, 3475-3483.

Karp, A. (1991). On the current understanding of somaclonal variation. *Oxf. Surv. Plant Mol. Cell Biol.* **7,** 1-58.

Linsdey, K., Wei, W., Clarke, M. C., Mcardele, H. F., Rooke, L. M. and *J. F. Topping* (1993). Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. *Transgenic Res.* **2(1)**, 33-47.

Linsmaier, E. M. and *F. Skoog* (1965). Organic growth factor requirement for tobacco tissue cultures. *Phys. Plant.* **18**, 100-128.

Masoud, S. A., Johnson, L. B., White, F. F. and *G. R. Reeck* (1993). Expression of a cysteine proteinase inhibitor (oryzacystatin-I) in transgenic tobacco plants. *Plant Mol. Biol.* **21**, 655-663.

Meiyalaghan, S., Barrell, P. J., Jacobs, M. E. and *A. J. Conner* (2011). Regeneration of multiple shoots from transgenic potato events facilitates the recovery of phenotypically normal lines: assessing a cry9Aa2 gene conferring insect resistance. *BMC Biotechnology.* **11**, 93.

Meiyalaghan, S., Pringle, M. J., Barrell, J. P., Jacobs, M. E. J. and *J. A. Conner* (2010). Pyramiding transgenes for potato tuber moth resistance in potato. *Plant Biotechnol. Rep.* **4,** 293-301.

Mitten, D. H., Horn, M., Burrell, M. M. and *K. S. Blundy* (1990). Strategies for potato transformation and regeneration, In: *The molecular and cellular biology of the potato* (Eds. W. R. Belknap, M. E. Vayda, and W. D. Park), 181-191. Wallingford, United Kingdom.

Munger, A., Goulet, C., Vaillancourt, L-P., Schluter, U., Kiggundu, A., Kunert, K., Goulet, M. C., Coenen, K. and *D. Michaud* (2010). Constitutive expression of endogenous defense proteins in transgenic potato lines expressing the Cys protease inhibitor corn cystatin II. *Asp. Appl. Biol.* **96,** 157-164.

Murashige, T. and *F. Skoog* (1962). A revised medium for rapid growth and bioassays with tobacco tissues cultures. *Phys. Plant.* **15**, 473-479.

Prins, A., van Heerden, P. D. R., Olmos, E., Kunert, K. J. and *C. H. Foyer* (2008). Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. *J. Exp. Bot.* **59**, 1935-1950.

Richter, R., van Damme, J. and *J. Wirz* (2011). Unintended phenotypic effects of single gene insertions in potatoes – assessing developmental dynamics and leaf morphology. *Elemente der Naturwissenschaft.* **95**, 9-24.

Sala, F., Arencibia, A. D., Castiglione, S., Yifan, H., Labra, M., Savini, C., Bracale, M. and *P. Pelucci* (2000). Somaclonal variation in transgenic plants. *Acta Horticulture.* **530,** 411-419.

Schluter, U., Benchabane, M., Munger, A., Kiggundu, A., Vorster, J., Goulet, M. C., Cloutier, C. and *D. Michaud* (2010). Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective. *J. Exp. Bot.* **61**(15), 4169-4183.

Senthilkumar, R., Cheng, C. P. and *K. W. Yeh* (2010). Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. *Plant Biotechnol. J.* **8**, 65-75.

Solomon, M., Belenghi, B., Delladonne, M., Menachem, E. and *A. Levine* (1999). The involvement of cystein proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell* **11**, 431-443.

Torres, J., Villanueva, D., Cadavid-Restrepo1, G. and *R. Arango* (2012). Phenotypic evaluation of two transgenic potato varieties for control of *Tecia solanivora. Agronomía Colombiana.* **30**(1), 7-14.

Van der Vyver, C., Schneidereit, J., Driscoll, S., Turner, J., Karl, K. and *C. H. Foyer* (2003). Oryzacystatin I expression in transformed tobacco produces a conditional growth phenotype and enhances chilling tolerance. *Plant Biotechnol. J.* **1**, 101-112.

Wilson, A., Jonathan, R., Latham, R. and *A. R. Steinbrecher* (2006). Transformation-induced mutations in transgenic plants: Analysis and biosafety implications. *Biotechnol. Genet. Eng.* **23**, 209-234.

Wu, R., Guo, L. W., Wang, X. R., Wang, X. L., Zhuang, T. T., Jihong, L. C. and *B. Liu* (2009). Unintended consequence of plant transformation: biolistic transformation caused transpositional activation of an endogenous retrotransposon Tos17 in rice ssp. japonica cv. Matsumae. *Plant Cell Rep*. **28**, 1043-1051.

Zeller, S. L., Kalinina, O., Brunner, S., Keller, B. and *B. Schmid* (2010). Transgene x environment interactions in genetically modified wheat. *PLoS One.* **5**(7), e11405.