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Transgenic reduction of cytokinin levels in roots inhibits root-sprouting in *Populus*

Dear Editor,

Summary paragraph: Root sprouts—the formation of new shoots from roots—is an important mechanism for local gene flow from poplar (*Populus* spp.). An effective strategy to reduce root sprout formation could therefore help to ensure containment during field research and commercial deployment of poplar when grown as exotic or transgenic forms. We used a flavonoid glycosyltransferase gene promoter from *Scutellaria barbata* (*SbUGT*) to drive the expression of *AtCKX2,* a cytokinin oxidase from *Arabidopsis* that converts active to inactive cytokinins in roots of poplar. In the greenhouse, *SbUGT::AtCKX2* transgenic plants exhibited a similar shoot growth habit, but had enhanced root growth and fewer root sprouts, compared to the wild type control and transgenic events with low transgene expression in roots. Under field conditions, the transgenic trees also had similar growth habits and stem growth rates that were not statistically different from wild type trees over three years. Removal of trunks generally induced high rates of root sprouting; however, in selected *SbUGT::AtCKX2* transgenic poplar events there was an absence or fewer root sprouts compared to wild type trees, consistent with the greenhouse results. Our study demonstrates that the *SbUGT::AtCKX2* gene can effectively inhibit root sprouting of poplar trees under field conditions, and thus may provide a useful tool to address concerns associated with local tree and transgene spread.

Poplar (*Populus* spp.) includes woody crops that are important for a variety of uses and products, including pulp, bioenergy, wood, bioremediation, wind protection, and agroforestry. Although transgene-free methods have been developed using the CRISPR/Cas system to improve perennial plants such as poplar, transgenic technologies are still powerful means to create new traits (Chen et al., 2018). Transgenic technology has been effectively used to produce poplar trees with a variety of useful traits, including herbicide resistance, biotic and abiotic tolerance, improved growth rate, higher nutrient use efficiency, and improved processing and end-use characteristics (Yang et al., 2009; Chang et al., 2018). Although many transgenic field trials have been carried out in the US, China, and elsewhere, no transgenic trees have been commercially adopted to our knowledge (Li et al., 2016; Klocko et al., 2018).

For most tree species, gene flow primarily occurs through seed and pollen (DiFazio et al., 2012). A number of molecular confinement strategies have been reported that may block seed and pollen-mediated transgene flow in tree species (Klocko et al., 2018). We have also recently demonstrated that an *AGAMOUS* intron-driven expression of a cytotoxin gene can be used to produce flowerless tobacco, and this technique may also be effective in reducing pollen- and seed-mediated transgene flow in poplar (Li et al., 2016). Further, we have reported a transgene deletion technology called the Gene Deletor that might be useful in eliminating transgenes from pollen and seeds of poplar, possibly obviating the need for containment (Luo et al., 2007).

For many plant species, including poplar trees, root sprouts are another vehicle for spread of transgenes. In general, root sprouting is common in poplar in both natural and commercial plantings (Jean et al., 2019). When the trunk of a poplar tree is removed or damaged, sprout bud primordia from roots will develop and grow to produce shoots. The ability to grow new ramets via root sprouting can be extensive and repetitive, making poplar difficult to eliminate from field

plantings. Also, after many years, poplar root sprouts can spread to form extensive clonal colonies, extending from many meters to kilometers (Wiehle et al., 2009; Stener et al., 2018). Further, traits, such as faster growth, improved water use efficiency and pest resistance, could create additional concerns if these genes were to spread to the native flora (Strauss et al., 2015). For small scale plantings, sprouts can be controlled by hand pruning and/or by repeated use of herbicides. However, for large scale plantations, it can be difficult and expensive to eliminate root sprouts entirely, making full compliance with regulatory requirements for transgenic field trials difficult.

The plant hormone cytokinins play a role in root sprouting. Exogenous cytokinins may stimulate root sprouting in poplar (Frey et al., 2003). Over-production of cytokinins in transgenic plants using the *Agrobacterium ipt* gene resulted in enhanced adventitious shoot development from both callus tissues and unwounded plants (Smigocki and Owens, 1988). A high ratio of exogenously applied cytokinin/auxin enhances root sprouts in *Emmenopterys henryi* (Guo et al., 2017), and the positive effects of auxin depletion on shoot initiation in citrus was shown to be mediated by cytokinins (Hu et al., 2017). Overexpression of a cytokinin oxidase (*CKX*) gene has also been shown to affect shoot development. Cytokinin deficient transgenic *Arabidopsis*, tobacco, and orchid that over-expressed *CKXs* showed improved root growth but reduced shoot initiation and growth (Werner et al., 2003; Werner et al., 2010). *CKX* overexpression also led to fewer shoots and flowers (Werner et al., 2003), while overexpression of a *CKX* gene driven by a root-predominant promoter caused few observable effects on shoot growth and development in both *Arabidopsis* and tobacco (Werner et al., 2010; Li et al., 2017).

In this study, we first used the root-dominant promoter *SbUGT* (Chiou and Lin, 2010; Li et al., 2017) to control the expression of the *GUS* reporter gene and *CKX2*-coding sequence. The *SbUGT::GUS* fusion gene was predominantly expressed in roots of transgenic poplar trees (Supplemental Fig. S1a). We were able to detect *CKX2* gene expression in roots for all 121 *SbUGT::CKX2* (*CKX2*) transgenic poplar events produced, of which 92 insertion events (76%) had no detectable *CKX2* expression in shoots (Supplemental Fig. S1b); the remaining 29 events (24%) exhibited modest but visible *CKX2* expression in shoots. More than 80% (98 events) showed visibly similar shoot growth compared to wild type. The remaining 20% showed reduced shoot growth similar to constitutive overexpression of a *CKX* gene, presumably due to leaky expression of the *CKX2* gene in leaf and stem tissues (Werner et al., 2003). The root-predominant *SbUGT* promoter sequence has been used previously in our lab to control the expression of *GUS* reporter gene, *iaaM* and *CKX2* genes in roots of tobacco (Li et al., 2017). Here, we further demonstrate that the *SbUGT* promoter is predominantly expressed in roots of poplar. The *SbUGT* promoter appears to be a novel root predominant promoter sequence in poplar and thus may provide a useful tool for transgene-mediated improvement of root growth and other characteristics in poplar and related species.

Auxin has been proposed as a negative regulator, and cytokinin as a positive regulator, of root sprout development (Jean et al., 2019). We analyzed cytokinin and auxin concentrations in roots of wild type and two representative events, 117 and 97. Cytokinin (zeatin riboside) levels in both transgenic events were reduced by 46% and 88%, respectively, relative to the levels in wild type control tissues (Supplemental Fig. S1c). Also, indole acetic acid (IAA) levels in both transgenic poplar events 117 and 97 were significantly reduced by 51% and 30% of the wild type poplar

roots, respectively (Supplemental Fig. S1d). These results suggest that the reduction in cytokinin level in root tissues is caused by root-predominant *CKX* overexpression, and thus contributes to the inhibition of sprout development. It has also been proposed that the auxin to cytokinin ratio may play an important role in regulating root sprouting (Frey et al., 2003; Guo et al., 2017). For event 97 which had no sprouts observed, we found a significant increase in the auxin to cytokinin ratio when compared to that of the wild type roots (i.e., 242 vs. 42). On the other hand, for event 117 that also had no sprouts, the ratio of root auxin to cytokinin did not appear to increase compared to wild type (i.e., 38 vs 42). It is therefore unclear whether the auxin to cytokinin ratio plays a significant role in regulating root sprout development. Further analyses of the concentrations of the different types of active cytokinins would improve our understanding of their effects in regulating root sprout formation of poplar trees, and should be a priority for further studies.

After three months of growth in a greenhouse, the trunks of wild type controls and 98 *CKX2* transgenic events with similar shoot growth were removed at 2 inches above the soil surface and the number of root sprouts of each tree was recorded six months later. We observed that 57% (56 events) of the *CKX2* transgenic poplar trees produced no root sprouts, 26% (25 events) produced a reduced number of sprouts, and 17% (17 events) produced a similar number of sprouts as the wild type poplar trees (Supplemental Fig. S2, Supplemental Table S1). We performed reverse transcription quantitative PCR (RT-qPCR) analyses for representative transgenic poplar events: three events with no sprouts (Events 97, 28, 117), four events with fewer sprouts (Events 75, 27, 51, 101), and three events with numbers of sprouts similar to those of wild-type plants (Events 9, 49, 125). Supplemental Table S2 shows that relatively high *CKX* expression was detected in the roots of three events with no sprouts, medium expression levels were detected in four events with reduced numbers of sprouts, and events that produced sprouts similar to that from wild-type poplar plants had less than 0.5% of the expression levels of the *UBQ* gene in roots. It therefore appears that the expression level of the *CKX* gene in roots is negatively correlated with the number of root sprouts.

We then selected five representative *CKX2* transgenic poplar events to retest the response to trunk removal under both greenhouse and field conditions. These *CKX2* transgenic poplar events included three (97, 28, and 117) that produced no sprout shoots and two (27 and 101) that produced fewer sprout shoots relative to the wild type. In the greenhouse, each event was propagated and grown for one year before removal of their trunk (Fig. 1a). Wild type trees produced 2.6 sprout shoots per plant on average. The propagated replicates of *CKX2* transgenic events 27 and 101 produced fewer root sprouts than the wild type plants under the same conditions, 1.0 and 1.2 per plant on average, respectively (Table 1). Further, all vegetatively propagated replicates of *CKX2* transgenic events 28, 97 and 117 failed to produce any root sprouts under greenhouse conditions (Fig. 1b and c). The sprouting abilities of these propagated poplar plants were consistent with those of their parental plants. Plants evaluated under field conditions were allowed to grow for three seasons before trunk removal (Fig. 1d). One growth season after the removal of their trunks, wild type poplar trees produced 5.0 root sprouts per plant on average, while events 27 and 101 produced 1.8 and 2.7 sprouts, respectively. Further, all replicate plants of events 28, 97, and 117 produced no sprouts (Table 1). These results are consistent with the observations in the greenhouse studies (Fig. 1e-h). Our results demonstrate that the *SbUGT::AtCKX2* gene can effectively inhibit root sprouting of poplar trees under both

greenhouse and field conditions.

Five selected representative transgenic events exhibited similar shoot growth as the wild type controls in the greenhouse (Fig. 2a-d). Events 28, 97, 101, 117 had significantly enhanced root biomass when compared to wild type, while event 27 had similar root biomass as wild type (Fig. 2e). Under field conditions, no significant differences were observed in the biomass index (estimated using height×diameter^2) between the average of all tested *CKX2* transgenic events and wild type poplar after three years (Fig. 2f). The effects of *CKX2* overexpression in roots on tree physiology warrant further study before commercial use. The enhanced root growth observed may be advantageous or cause unintended effects, for example, on drought tolerance or long term stem productivity. These effects are likely to be highly soil and environment dependent, and thus are difficult to judge from a single field study. Further, we have not studied whether *CKX2* overexpression also affects propagation from stem cuttings-the main method by which poplars are amplified for commercial use. Hopefully, by selecting events with optimal root expression, or ultimately using a more cell-specific root dominant promoter, a system can be developed which strongly suppresses root sprouts without any undesired physiological effects.

In summary, we have shown that overexpression of *CKX2* driven by a root predominant promoter *SbUGT* resulted in reduced levels of cytokinins in roots of poplar. We have further shown that under both greenhouse and field conditions some *SbUGT::CKX2* transgenic poplar events exhibited no visible alterations of shoot growth, but did exhibit enhanced root growth, compared to wild type trees. Finally, we have demonstrated that some of the same *SbUGT::CKX2* poplar events did not produce root sprouts under both greenhouse and field conditions. These results suggest that this technology may be useful to eliminate or reduce sprout shoot development in poplar, as well as other root sprouting plant species. The root sprout repressing approach described here may provide a useful tool to address sprout-mediated gene flow and related problems for transgenic poplar and other woody plant species.

Supplemental Data

The following supplemental materials are available.

Supplemental Materials and Methods.

Supplemental Figure S1 Root-dominant expression of *CKX2* resulted in reduced cytokinins content in roots.

Supplemental Figure S2 Sprouts number of nine wild type (WT) ramets and 98 independent transgenic events.

Supplemental Table S1 Data of sprouts number of wild type (WT) ramets and independent transgenic events.

Supplemental Table S2 Relative expression levels of the *CKX* gene in representative transgenic poplar plants.

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One Sentence Summary: An analysis of a root sucker repressing technology for poplar plants which reduces root cytokinin contents.

Footnotes

- The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is:

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- Y.L. Z.H. and W.L. conceived and designed the research or the manuscript organization and writing; W.L., L.Z., H.Y., J.C., X.W. W.Z., C.F., and Y.C. conducted the experiments; E.M. was responsible for endogenous hormone analysis and manuscript editing; R.J.M. provided suggestions on experiments and manuscript editing; W.L., Y.L., S.H.S., Z.H., and L.Z wrote and edited the manuscript. All authors read and approved the article.

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Figure 1 An absence, or reduced, sprouting in transgenic poplars in the greenhouse and field. (a) 4-month-old potted, greenhouse-grown plants. (b-c) In the greenhouse, repressed root sprouting was observed in *CKX2* transgenic event 97 (c) when compared to wild type (b). (d) Images of the field test, the photo was taken in July 2016. (e-h) *CKX2* transgenic events 101 (f), 27 (g) and 117 (h) exhibited no or fewer root sprouts than wild type (e) plants one growth season after the excision of the trunk in the field. Photos were taken in November of 2017. The trunks are inside the yellow colored circles. Bar = 12 cm.

Figure 2 Shoot and root growth in *CKX2* compared to wild type poplar. (a-b) No morphological differences were observed between wild type (a) and transgenic poplar plants (b) after visual inspection after 3-months of growth in the greenhouse. (c-e) In greenhouse, overexpression of the *CKX2* gene in roots of poplar gave similar shoot growth (c,d) but significantly enhanced root growth (e) when compared to wild type. (f) No statistically significant differences in stem biomass index (estimated using height×diameter^2) were observed between transgenic and wild type plants after 3-years of growth (2013-2016) in the field according to Student's t-test with the pooled variance at P = 0.05. Bars represent standard errors.

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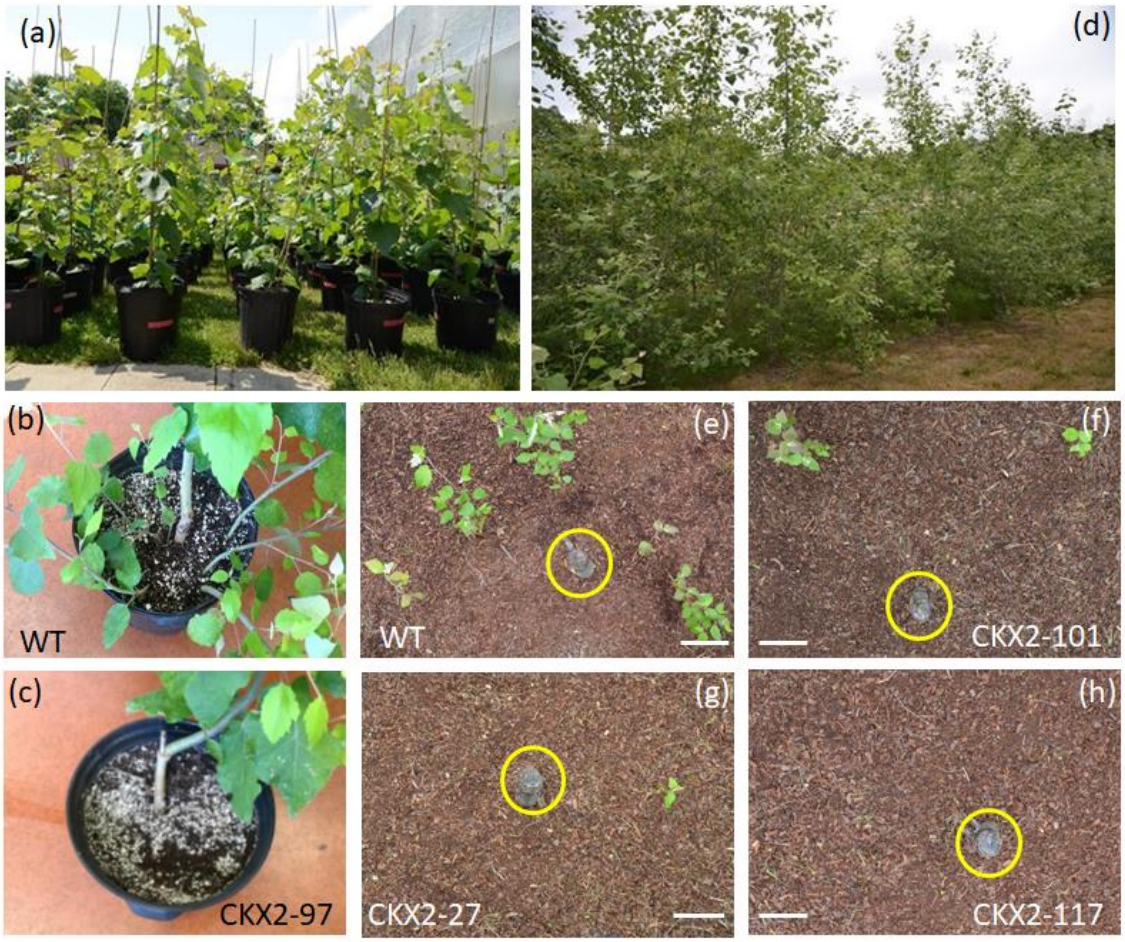
Table 1 Rate of root sprouting for *CKX* transgenic and wild type poplar in the greenhouse and field.

Genotype	In Greenhouse	In Field
WT	2.6 ± 0.4	5.0 ± 0.9
Event 27	1.0 ± 0.3	1.8 ± 0.5
Event 101	1.2 ± 0.3	2.7 ± 0.3
Event 28	0*	0*
Event 97	0*	0*
Event 117	0*	0*

Data in the table represent average number of suckers per event. At least six replicates were used for wild type and each transgenic event. Differences determined by pairwise, two-sided chi-square tests on the data before averaging. Asterisks (*) represent significant differences between wild-type and *CKX2* transgenic event for either greenhouse or field evaluation ($P<0.05$).



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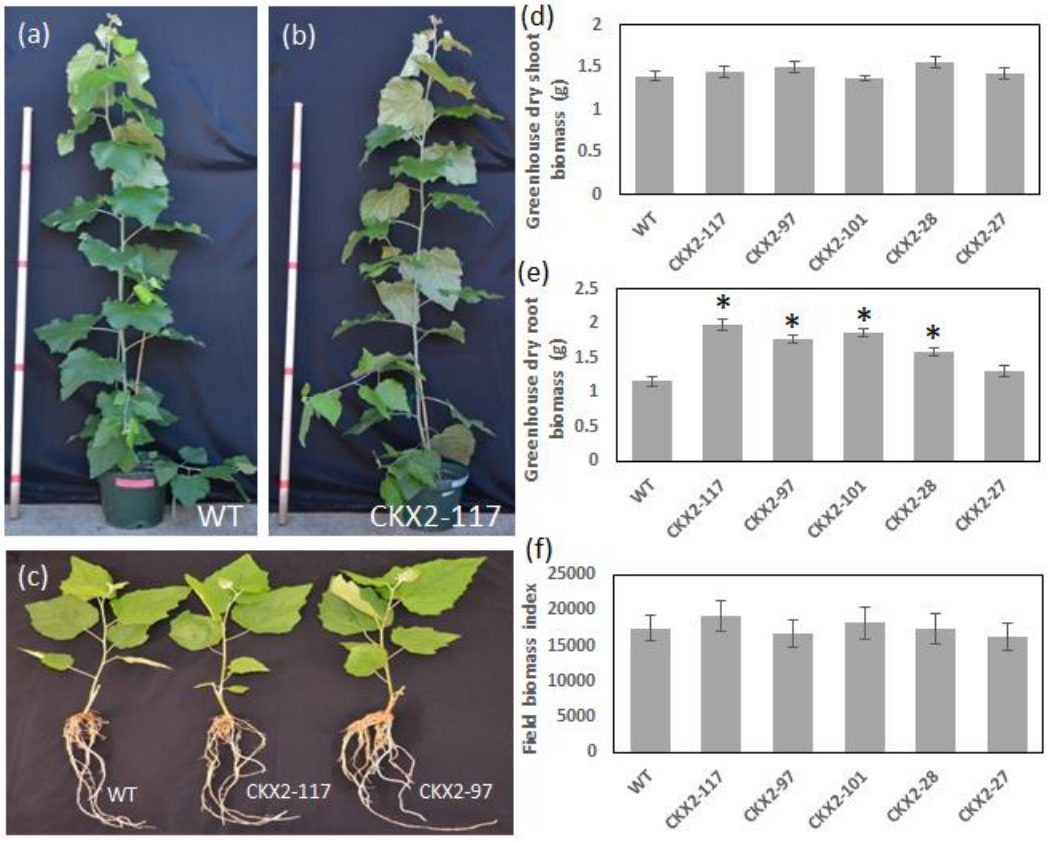

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