Exploring the Mechanism of Potassium Chlorate-Induced Flowering in *Dimocarpus longan*

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Abstract

Flowering is the vital stage of plant development since it is the precursor to fruit and seed production as well as the vehicle for genetic improvement by breeding. Understanding flower induction in tree crops is often difficult since trees are a long-term perennial crop that is subject to environmental and cultural changes over multiple seasons of growth. Unlike temperate fruit trees, where growth, dormancy and break of dormancy is determined by the seasons, subtropical and tropical fruit trees rely on more subtle changes in rainfall, temperature or nutrient availability. The discovery of potassium chlorate induced flowering in longan provides an excellent opportunity to investigate the mechanism of flowering in longan. Preliminary experiments suggest that chlorate inhibits nitrate reductase activity in longan trees treated with potassium chlorate, however there is no significant difference in the total nitrogen content and carbon:nitrogen (C:N) ratio in potassium chlorate treated and untreated trees. In addition to potassium chlorate, sodium chlorite and sodium hypochlorite are also able to effectively decrease nitrate reductase activity and induce flowering of longan trees. To identify genes that may be involved in potassium chlorate induced flowering, we created a suppressive subtractive hybridization (SSH) library to longan cDNAs that are differentially expressed in vegetative buds or floral buds induced by potassium chlorate.

INTRODUCTION

Longan trees are commonly grown in many subtropical and tropical countries with majority of the production in Thailand, Taiwan, China and Australia (Menzel et al., 2002). In the United States the majority of the production is in Florida, Hawaii and California. In 1998, the United States produced 1.4 million pounds of longan with an estimated value of $2.8 million (Mossier and Nesheim, 2002). In Hawaii, longan production was valued at $425,000 for 125,000 pounds sold in 2004 which represents a 10% increase in production from 2003. Future production is expected to increase since half of the trees are not bearing fruit (Hawaii Agriculture Statistics, 2005).

The discovery of potassium chlorate induced flowering by Dr. Chung-Ruey Yen of the National Pingtung University of Science and Technology in Taiwan has overcome the problem of alternate bearing and enabled growers to produce off-season longan. Dr. Yen was able to associate the use of fireworks during religious ceremonies with off-season production of longan flowers on trees near the temples in Taiwan. By applying discarded soil from a fireworks factory to the soil of longan trees, 5% of the canopy flowered within 110 days, suggesting that the gunpowder from fireworks may contain the active ingredient for flower induction. The chemicals of gunpowder were applied as single ingredients or combinations of ingredients to two varieties of longan. Potassium chlorate alone or in combination with other chemicals was found to increase the percentage of flowers to the same extent as gunpowder, suggesting that potassium chlorate was the chemical controlling longan flowering (Yen, 2000; Yen et al., 2001). Since this discovery, potassium chlorate is used to induce off-season flowers and fruits in longan trees worldwide (Choo, 2000; Manochai et al., 2005).

In agriculture, chlorate has been extensively used as a herbicide to control...
problematic weeds such as bindweed (Latshaw and Zahnley, 1927; Neller, 1930; Loomis et al., 1933). Investigation of the mechanism of chlorate by Borje Åborg in 1947 suggested that the toxicity of chlorate was caused by reduction of chloride to chlorite and hypochlorite and that this reduction was carried out by nitrate reductase (reviewed by LaBrie et al., 1991). As predicted by Åborg, the majority of plant nitrate reductase enzymes reduces chlorate to the toxic chlorite. Sodium hypochlorite, the active ingredient in bleach and calcium hypochlorite, and the active ingredient in swimming pool cleaner, can also induce flowering in longan although not as effectively as potassium chlorate (Sritontip et al., 2005).

Carbon to nitrogen (C:N) ratio is an important physiological factor in flowering. High carbohydrates and low nitrogen result in a high C:N ratio which favors flowering and high nitrogen results in a lower C:N ratio which favors vegetative growth. Recent studies suggest photoperiods conducive to flowering lead to a high C:N ratio in the phloem which may be important to the floral transition in the meristem of *Sinapsis alba* and *Arabidopsis thaliana* (Corbesier et al., 2002).

In addition to the total nitrogen content in the plant, the form of nitrogen is also important for the induction of flowering. In mango, flower induction is achieved by spraying the vegetative shoot with potassium nitrate (Bondad and Apostol, 1979; Davenport, 2000). Flower initiation in apples can be achieved by exposure to ammonium, polyamines or sub-lethal levels of simazine (Grasmanis and Edwards, 1974; Rohozinski et al., 1986). The herbicide simazine inhibits the photosynthetic electron transport resulting in an increased nitrate reductase activity, and accumulation of ammonium and arginine, which are precursors to polyamines (Rohozinski et al., 1986). The efficiency of flower induction of apples by ammonium varies and is dependent upon the rootstock cultivar (Gao et al., 1992). The polyamine, putrescine was found to parallel an increase in ammonium and arginine during the development of flowers in the ‘Washington’ navel orange (Sagee and Lovatt, 1991).

Here we describe the effect of potassium chlorate, sodium chlorite, sodium hypochlorite and sodium hypochlorite plus potassium nitrate on nitrate reductase activity and flowering of longan trees. The C:N ratio and ammonium nitrogen content was compared in trees treated with potassium chlorate, 4 and 7 weeks after treatment. Genes isolated from a suppressive subtractive hybridization (SSH) cDNA library constructed from vegetative and potassium chlorate induced floral buds is discussed.

### MATERIALS AND METHODS

#### Nitrate Reductase Activity

Analysis of nitrate reductase activity was based upon Scheible et al. (1997). Leaf samples of fully expanded leaflets closest to the growing meristem were collected at the same time of day, weighed and frozen in liquid nitrogen. Frozen samples were ground to a fine powder with a mortar and pestle and placed in extraction buffer. Assays were conducted at 30°C and samples were taken at 15-minute intervals over a period of 1 hour to ensure linearity of the samples. The color reaction was read on a spectrophotometer at an absorbance of 540 nm. Three replicates were taken from each tree.

#### Potted ‘Biew Kiew’ Plants

‘Biew Kiew’ longan trees propagated by air layers were planted in medium consisting of an equal mixture of soil, macadamia husks and black volcanic cinder in 19 L pots. Plants were grown in a covered greenhouse and irrigated twice a week. Plants were fertilized with granular Miracle Gro (15-30-15) (15 ml/3.79 L) every week. Three trees of each treatment were either not treated (control) or treated with 5 g/pot of potassium chlorate (KClO₃), 5 g/pot of potassium nitrate (KNO₃) or 5/g pot of both potassium chlorate and potassium nitrate (KClO₃/KNO₃). Two samples from each tree were harvested two weeks after treatment with potassium chlorate or potassium nitrate and samples were monitored over time to insure linearity of the assay.
Effect of Chlorate, Chlorite, Hypochlorite and Nitrate on Nitrate Reductase Activity and Flowering

Four-year-old Dimocarpus longan ‘Egami’ and ‘Biew Kiew’ trees grown at the University of Hawaii, Waiakea Agriculture Research Station were used for this study. The station is 9.7 km outside of Hilo, with an elevation ranging from 175 to 227 m. Mean maximum and minimum temperature is 28 and 16°C, respectively. Annual rainfall averages 444.5 cm and is most abundant during October to February. The soil consists of an extremely stony Papai muck with organic soils formed over mostly fragmental a‘a lava.

On September 15, 2004, a total of 3 longan trees, 2 ‘Egami’ and 1 ‘Biew Kiew’ trees, were randomly treated with one of six treatments: 1) no treatment (control), 2) 300 g/tree KClO3, 3) 300 g/tree sodium chlorite (NaClO2), 4) 300 g/tree potassium nitrate (KNO3) applied as a broadcast under the tree canopy, 5) 7.57 L bleach (5.25% sodium hypochlorite) or 6) 300 g/tree KNO3 plus 7.57 L bleach (5.25% sodium hypochlorite) applied as a soil drench under the tree canopy. Leaf samples were collected and assayed for nitrate reductase activity, as described above, for two consecutive weeks after the treatment. Trees were monitored weekly for flowering, which commenced from October 22 and continued until December 10, 2004. Flowering was measured as a percentage of the flowering terminals divided by the total number of terminals. Data was analyzed using ANOVA using SigmaStat (Systat Software, Point Richmond, CA).

C:N Ratio and Ammonium Nitrogen Analysis

Seven-year-old Dimocarpus longan ‘Egami’ trees grown at the USDA, ARS, Pacific Basin Agricultural Research Center (PBARC), Tropical Plant Genetic Resource Management Unit in Hilo, Hawaii located at the University of Hawaii, Waiakea Agriculture Research Station were used for this study. Growing conditions are described above. Trees were divided into two groups. The first group of three trees was treated with 300 g of granular potassium chlorate (KClO3) evenly spread below the outer edge of the canopy of the tree on June 15, 2004, and, the second group of three trees was untreated control located randomly throughout the plot. Inflorescence buds were visible on KClO3 treated trees after 6 weeks and flowers were visible 9 weeks after application, while untreated trees remained vegetative.

Leaf samples were collected from the middle leaflets on the third compound leaf from the tip (Xinghui et al., 1986). A total of 8 leaflets were collected from each tree. Samples were wiped clean with distilled water and dried for 2 to 3 days in a circulating drying oven set at 70°C. Samples were sent to Micro Macro International (Athens, Georgia) for C:N and ammonium nitrogen (Protein N%) analysis.

RESULTS AND DISCUSSION

Similar to reports of potassium chlorate reducing the activity of nitrate reductase in Arabidopsis (LaBrie et al., 1991) reduced nitrate reductase activity was observed in 1-year-old potassium chlorate treated ‘Biew Kiew’ longan trees grown in pots (Fig. 1). Potassium nitrate treatments slightly increased nitrate reductase activity compared to control plants, however, due to the large variability of nitrate reductase activity in untreated control plants, this was not significant. Nitrate reductase activity was reduced to 27% and 10% in potassium chlorate and potassium chlorate/potassium nitrate treated trees, respectively, relative to control. The dramatic reduction of nitrate reductase in potassium chlorate/potassium nitrate treated longan plants was not expected since the K_m of nitrate reductase for chlorate is 50 to 100 times greater than nitrate (LaBrie et al., 1991). However, the enzyme kinetics for longan nitrate reductase is unknown. Another explanation for this result may be that the presence of nitrate in the cells increases the susceptibility of the nitrate reductase enzyme in the cell to the toxicity of chlorate ion (LaBrie et al., 1991).

Since the plants in the pots did not flower, we repeated the experiment with field-grown plants treated with potassium chlorate (KClO3), potassium nitrate (KNO3), sodium...
chlorite (NaClO₂), bleach (NaOCl) and bleach plus potassium nitrate. Chlorite and hypochlorite result from reduction of chlorate by nitrate reductase. The addition of nitrate to chlorate further reduced nitrate reductase. Therefore, we investigated if chlorite and hypochlorite also reduce nitrate reductase activity similar to chlorate and if reducing nitrate reductase activity with the addition of nitrate to NaOCl will also enhance flowering.

Nitrate reductase activity was assayed two consecutive weeks after treatment (Fig. 2). Interestingly, all treatments except nitrate treated trees, resulted in higher nitrate reductase activity than control on week 1 but were reduced in week 2. In Arabidopsis plants grown without nitrate, the expression of the nitrate reductase gene is induced by chlorate (LaBrie et al., 1991). This may explain the increase in nitrate reductase activity observed in longan. All treatments including KClO₃, NaOCl and NaClO₂ reduced nitrate reductase activity. Similar to the application of KClO₃/KNO₃ treatment, in the potted longan plants, nitrate reductase activity of the NaOCl treatments was further reduced by the addition of KNO₃. Trees treated with NaClO₂ showed the greatest reduction of nitrate reductase activity and this is probably due to the fact that chlorite is the product that inhibits nitrate reductase activity while chlorate must first be reduced to chlorite (LaBrie et al., 1991). All the trees with the exception of control and KNO₃ treated trees produced flowers. The flowering percentages were control and KNO₃ (0%), KClO₃ (97.8%), NaOCl (76%), NaOCl plus KNO₃ (99.4%) and NaClO₂ (93.4%) suggesting chlorate, chlorite and hypochlorite can all effectively induce longan flowering. ANOVA analysis and mean separation by Tukey’s multiple range test indicated that all treatments were significantly different (P<0.05) relative to control and KNO₃ but not significantly different relative to each other. Sodium hypochlorite was effective in promoting flowering, which differs from previous reports that this treatment was less effective than KClO₃ (Sritontip et al., 2005). This may be attributed to the a’ a soil that is mainly porous rock that would allow for greater penetration of the hypochlorite to the roots. Application of NaOCl to the roots in a clay rich soil resulted in reduced levels of flowering (Nagao et al., 2003).

Our results indicate that nitrate reductase activity is lower in potassium chlorate treated longan trees. In ‘Do’ longan plants treated with potassium chlorate in Thailand, total nitrogen content was higher than in untreated controls (Wangsin and Pankasemsuk, 2005). This suggests that although the reduction of nitrate to nitrite may be reduced, other ions of nitrogen such as ammonium or nitrite may be higher in potassium chlorate treated plants. Similar to the results obtained by Wangsin and Pankasemsuk (2005) we could not detect a significant difference in C:N ratio between control and plants treated with KClO₃, 4 weeks (control 30:1 and KClO₃ treated 29:1) and 7 weeks (control 29:1 and KClO₃ treated 28:1) after treatment. Ammonium nitrogen was not significantly different between control and treated plants 4 (control 11.03 protein N % and KClO₃ treated 11.33 protein N %) and 7 weeks (control 11.73 protein N % and KClO₃ treated 11.54 protein N %) after treatment.

In this investigation we have found that chlorate, chlorite and hypochlorite all reduce the levels of nitrate reductase activity and are able to induce flowering. We could not detect if the level of reduction is correlated with flowering since all treatments promoted flowering equally well. However, nitrogen levels as well as C:N ratio were not significantly different in KClO₃ treated trees before flowering. Therefore, chlorate and the reduction products of chlorate, including chlorite and hypochlorite may cause an alternative pathway for floral induction in longan besides modification of total nitrogen levels. Nitric oxide in Arabidopsis suppresses flowering by modifying the amplitude of the diurnal rhythms of the circadian clock and the expression of floral regulatory genes. Nitrate reductase has been implicated in the conversion of nitrate to nitric oxide and a plant nitric oxide synthase has been identified to convert arginine to produce nitric oxide (Crawford and Guo, 2005).

A screen for differentially expressed eDNA from vegetative buds and potassium chlorate induced floral buds isolated 65 uniquely expressed genes. Shoot and floral
meristem development associated genes were identified in the screen, including homologs of Protodermal Factor 1 (PDF1), SHEPHERD and PISTILLATA. Although, genes associated with carbon metabolism and developmental regulation of flowering (i.e. fructose 1,6 bisphosphate aldolase and pyruvate dehydrogenase complex) were isolated in this screen, we did not isolate genes directly associated with nitrogen utilization (i.e. nitrate reductase etc.). The cDNA library was created from buds harvested in the later stages of floral development and transcriptional regulation of nitrogen utilization genes may occur earlier in the floral initiation process (i.e. immediately after chlorate treatment) (Matsumoto, 2006). Characterization of the nitrogen related genes and putative regulatory genes isolated in this screen may reveal possible mechanisms of action of potassium chlorate induced flowering and will be further investigated.

**Literature Cited**


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Fig. 1. Nitrate reductase activity, two weeks after treatment, of 1-year-old air layered ‘Biew Kiew’ longan plants treated with potassium chlorate (KClO₃), potassium nitrate (KNO₃) or both potassium chlorate and potassium nitrate (KClO₃/KNO₃) or left untreated (control). Bars represent standard error.

Fig. 2. Relative nitrate reductase activity of field grown ‘Egami’ longan plants treated with potassium chlorate (KClO₃), bleach (NaOCl), potassium nitrate (KNO₃), bleach plus KNO₃ or sodium chlorite (NaClO₂) one and two weeks after treatment. Bars represent standard error.