Scientia Horticulturae 159 (2013) 103–108

Contents lists available at SciVerse ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Field evaluation of transgenic pineapple (*Ananas comosus* (L.) Merr.) cv. 'Smooth Cayenne' for resistance to blackheart under subtropical conditions

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ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form 10 May 2013
Accepted 12 May 2013

Keywords:
Genetic modification
Genetic engineering
Gene silencing
Blackheart disorder
Polyphenol oxidase (PPO)
Transgenic pineapple
Field evaluation

ABSTRACT

A comparative analysis of transgenic pineapple lines transformed with a polyphenol oxidase (PPO) gene (*ppo*) and the untransformed cultivar 'Smooth Cayenne' was made from plants grown in a series of field trials under cool subtropical conditions in southeast Queensland. In the four field trials where blackheart was recorded, all of the control lines expressed blackheart on each occasion and exhibited the greatest incidence (50%) and severity (34%) of symptoms. Irrespective of the gene transfer method or the gene construct used, 38% of the lines produced were regarded as blackheart resistant, having no blackheart symptoms in two or more trials. Five blackheart resistant transgenic lines consistently performed as well as or better than control plants in terms of fruit characteristics and quality.

1. Introduction

Blackheart of pineapples is a physiological disorder that occurs when day/night temperatures drop below 25°/20 °C combined with low light conditions during fruit development (Abdullah et al., 2010). This is the case with winter production of 'Smooth Cayenne' pineapples in the subtropics but it can also occur during cool storage of harvested fruit. Because blackheart is a pulp disorder with no apparent external damage, identifying affected intact fruit is not possible. As a consequence blackheart is only detectable once the fruit has been cut open, creating problems with product reputation (Sanewski and Giles, 1997) and wastage during factory processing.

In pineapple, Stewart et al. (2001) have shown that the occurrence of blackheart is directly correlated with the oxidation of phenolic compounds by the enzyme polyphenol oxidase (PPO) and which can be controlled by a *ppo* gene. Attempts to control blackheart through selective breeding have been unsuccessful with 'Smooth Cayenne' due to sterility factors (Coppens d'Eeckenbrugge et al., 2011). In contrast to conventional breeding programs, the direct introduction of transgenes into potentially regenerative cells has proven to be a very effective and useful tool to modify undesirable characters or to introduce new desirable traits into crops.

This paper, for the first time, reports on the fruit yield and quality of transgenic pineapples engineered for blackheart resistance. A comparative analysis of transgenic pineapple lines and the conventional cultivar 'Smooth Cayenne' was made from plants grown in a series of field trials under cool subtropical conditions in southeast Queensland. Fruit were assessed for blackheart expression and PPO activity, as well as weight, size, percentage total soluble solids and titratable acidity.

2. Materials and methods

2.1. Plant materials

Transgenic pineapple lines were developed by both biolistics and *Agrobacterium tumefaciens*-mediated transformation (Ko et al., 2005, 2006, 2008) using *ppo* genes isolated from pineapple (Stewart et al., 2001). Constructs were designed containing the PINPPO1 gene in sense (*ppo*) and antisense-sense (*opp.ppo*) orientation and one containing a hairpin RNA with a spliceable intron (*opp.i.ppo*). In addition, 1 aminocyclopropane-1-carboxylic acid (ACC) synthase is the key enzyme responsible for increased ethylene production, which stimulates flowering, and is controlled by an *acc* gene (Trusov and Botella, 2006). Natural, uncontrolled

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0304-4238/$ – see front matter.
http://dx.doi.org/10.1016/j.scienta.2013.05.006

Fig. 1. (A) Variable plant size at time of planting; note plots of 5 plants per line are of similar size. (B) Remnants of bagging of flowers to prevent cross pollination of non-transformed pineapple sites. (C) Transgenic line 158-5.1 (left) showing a trace of blackheart compared to control fruit (right) under heavy blackheart selection pressure. Resistant lines showed no traces of blackheart. (D) Aberrant fruit with 'restricted neck' from transgenic line 91-18.

flowering is enhanced under the same adverse climatic conditions as those that trigger blackheart and is responsible for uneven fruit maturity leading to significant losses during crop harvest. Hairpin RNA silencing constructs targeting both *ppo* and *acc* genes were also designed (*opp.cca.i.acc.ppo*). 'Smooth Cayenne' is susceptible to blackheart and natural flowering and therefore non-transformed, micropropagated plants from axillary buds were used as control plants.

Putatively transformed *in vitro* plants were subjected to DNA analysis to confirm the presence of the gene of interest. PCR was performed on each individual plant with primers to detect the presence of the *ppo* gene and the *nptII* gene for geneticin (G418 sulfate) resistance selection (Ko et al., 2006).

The transformed plants were then hardened-off in the glasshouse and shadehouse prior to planting in the field. The process *ex vitro* took between six and 12 months. As new gene constructs were progressively designed and synthesized, lines containing initial constructs were superseded and replaced by newly produced lines necessitating a series of field trials to compare lines. Plant size and vigour varied and generally this was line dependent (Fig. 1A). The lack of vigour and small plant size of some lines meant that they did not respond to flower induction and consequently between 32 and 70% of the plants flowered to produce fruit for assessment from the trials.

Another field trial using conventional planting material was conducted between May 2005 and May 2007 and consisted of crowns originating from trial 1 fruit, so these plants represented a second generation *ex vitro* and were more uniform and robust. Consequently 95% of these plants flowered and produced fruit.

2.2. Field layout and induction of flowering

Planting was timed to occur in spring and approximately 12 to 15 months later plants in the field were sufficiently mature to be sprayed with Ethrel® growth regulator (Ethephon 480 g/L, Bayer Crop Science) to initiate uniform flowering. When flowering commenced after approximately three months, individual flowerheads were bagged to prevent cross-pollination of surrounding non-transformed pineapple plantings on the station, which was a biosecurity requirement (licence number DIR:028/2003) imposed by the Gene Technology Regulator (Fig. 1B). Ideally, early fruit development occurred during the colder months.

Four pineapple field trials were established at two sites over a five year period (2002–2007) (Table 1). All were completely randomised blocks with 5 plants per plot and surrounded with guard rows. The plots were arranged in rows with the plants positioned with a distance of 25 cm between them within the raised beds which contained two rows of plants (Fig. 1A). Harvest of fruit occurred weekly over a two month period. The total number of fruit assessed per line in trial 1 varied between 5 and 21, in trial 2 between 5 and 40, in trial 3 between 5 and 33, and in trial 4 between 7 and 51.

2.3. Blackheart assessment

Fruit was harvested when ¼ mature or less. The yellow external colour of the fruit from the base of the fruit up was indicative of the maturity rating. At harvest time, size (length, circumference