Gene and Genome Mélange in Breeding of Anthurium and Dendrobium Orchid

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Abstract

Both genome breeding (classical hybridisation) and molecular breeding approaches are used concurrently in our program for varietal development of Dendrobium and Anthurium as cut flowers and blooming potted plants. Two transgenic lines of anthurium 'Paradise Pink', engineered to produce the cecropin-like Shiva 1 lytic peptide, were able to significantly resist anthurium blight caused by Xanthomonas campestris pv. dieffenbachiae when compared to a standard resistant cultivar 'Kalapana'. However, disease severity could be significantly increased as well using the same transgene approach in a different genotype, 'Tropic Flame'. These lines were shown to be compatible with beneficial leaf-associated bacteria that can aid in suppressing blight, suggesting that use of GMO plants could be combined with beneficial bacteria to provide durable protection against anthurium blight disease. Blight resistance incorporated by hybridisation of A. andraeanum types with A. antioquiense also enhanced resistance, but the market-desired heart-shaped spathe form was difficult to recover. Both gene and genome breeding for resistance occurred in a comparable time frame of less than 10 years. Dendrobium orchid breeding has benefit greatly from molecular tools in understanding genetic control of flower colour. A chemical survey of Dendrobium species and hybrids showed lavender cyanidin and peonidin to be the predominant anthocyanidin and orange pelargonidin to be rare. Our cloning and characterization of key anthocyanin biosynthetic genes such as of dihydroflavanol 4-reductase enables more productive hybridisation strategies to be implemented.

INTRODUCTION

Anthurium and Dendrobium orchid are the two most important flower crops in Hawaii. Both classical and molecular breeding approaches are used concurrently for varietal development of cut flowers and blooming potted plants. Progresses achieved in breeding for bacterial disease resistance in Anthurium and for improved flower colour in Dendrobium are reported here with respect to using genome breeding (sexual hybridisation) or genetic engineering.

DISEASE RESISTANCE OF ANTHURIUM

Anthurium blight caused by the bacterium Xanthomonas campestris pv. dieffenbachiae (Xcd) has become a serious threat to the global anthurium industry. Genome breeding in Hawaii using Anthurium antioquiense has produced blight tolerant cut flowers (Kamemoto and Kuehnle, 1996). However, these lack the standard heart-shaped spathe due to the species influence and thus are less desirable for certain markets. Genes encoding peptide biocides have held promise for developing disease resistant crops. Cecropins and their synthetic counterparts are small (~4 kDa) lytic peptides that show high and broad potency against bacteria by interfering with inner and outer bacterial membranes (Boman, 1991).
Gene Transfer and Analyses

Two genetically distinct commercial cultivars that are susceptible to blight were selected to test these peptides in Anthurium. ‘Paradise Pink’ has a pink heart-shaped spathe and ‘Tropic Flame’ has a red tulip-type (cupped) spathe (Kamemoto and Kuehnle, 1996). These were engineered to produce the cecropin-like Shiva-1 lytic peptide in leaves, through Agrobacterium-mediated transformation with the plasmid pBPRS1 containing the PR1 secretion signal for Shiva-1, driven by the double CaMV 35S promoter (Kuehnle et al., 2001). Molecular analyses of clonally propagated transgenic lines by PCR, RT-PCR and ELISA showed that inserted Shiva-1 DNA was stably maintained and expressed in the transgenic lines over several years (Fig. 1; for details see Kuehnle et al., 2004, accepted).

Indirect competitive ELISA (Janssen, 1995) was used to estimate the level of Shiva-1 peptide in transgenic leaf tissues (Table 1). However, these values may be considerably lower (50-fold) than the actual values due to Shiva-1 degradation in the assay system which employed plant extracts but lacked addition of protease inhibitor (Florack et al., 1995). Adjusted by 50-fold they reflect the levels reported for grapevine (Li et al., 2001).

Disease Challenge and Analyses

Inoculation of Anthurium plants is summarized in Fig. 2 and described in detail in Kuehnle et al. (2004; accepted). Bioluminescent Xcd bacteria engineered to express the lux gene were employed to quantify disease progression in leaves, using X-ray film exposed to the leaves to detect the bacteria, for meaningful comparison with non-engineered controls as well as with the susceptible ‘Rudolph’ and resistant ‘Kalapana’ checks.

Disease progression was evaluated statistically in ‘Paradise Pink’ transgenic and non-transgenic plants (Fig. 3). Transgenic ‘Paradise Pink’ displayed significantly enhanced tolerance to bacterial blight (Pr>F=0.0006; n=8 to 10 replicates). Although complete resistance was not obtained, a blight tolerance level exceeding that of a resistant industry standard, ‘Kalapana’, is meaningful in this perennial crop.

Disease progression in ‘Tropic Flame’ transgenic lines was compared statistically to non-transgenic control plants (Fig. 4). One transgenic line of ‘Tropic Flame’, 1-16, showed significantly enhanced susceptibility to blight (Pr>F=0.0028; n=10) over the non-transgenic controls. Two other lines did not differ from the controls. Northern analysis results showed that ‘Tropic Flame’ line 1-16 had much lower transgene expression compared to line 1-9 (Fig. 5). This reduced transcription might explain the enhanced susceptibility of line 1-16, since low concentrations of Shiva-1 can stimulate bacterial growth before becoming inhibitory at higher concentrations. (Note that ‘Tropic Flame’ line 1-1 on Fig. 5 had a lower total RNA load compared to ‘Tropic Flame’ lines 1-9 and 1-16 and thus a fainter hybridisation signal; see Kuehnle et al. (2004; accepted) for details.)

Effect of Shiva-1 on Phyllosphere Residing Beneficial Bacteria

Transgenic and non-transgenic control plants were spray inoculated with a cocktail of four bacterial species originally isolated from Anthurium and which can serve a protective effect against bacterial blight (Fukui et al., 1999). Plants were bagged overnight and guttation fluid from the leaves (Fig. 6E) was collected in the morning. Dilution plate counts (Fig. 6F) of the four beneficial bacterial species residing in the host leaves after inoculation did not differ among the transgenic and wild type Anthurium lines. No inhibition or proliferation was observed in beneficial bacteria due to the production of Shiva-1 in transgenic plants.

CONCLUDING REMARKS FOR BREEDING DISEASE RESISTANCE IN ANTHURIUM

• Genome breeding using Anthurium antioquiense has produced blight tolerant cut
flowers. However, these lack the standard heart-shaped spathe due to the species influence and thus are less desirable for certain markets.

- Gene breeding using Shiva-1 significantly enhanced disease tolerance in one cultivar, ‘Paradise Pink’ with a standard heart-shaped spathe. Gene breeding using Shiva-1 unexpectedly enhanced the susceptibility to blight in another cultivar, ‘Tropic Flame’, of very different genetic make-up. Shiva-1 peptide quantity did not directly indicate increased tolerance to blight across different cultivars. However, reduced transcription levels of the transgene within a genotype were observed to enhance susceptibility to blight in ‘Topic Flame’. Down-regulation of a transgene under stressful environmental conditions might render an otherwise resistant transgenic cultivar more prone to blight infection. Therefore, considerable testing under field conditions is necessary to evaluate a transgene in Anthurium before release.

- Beneficial bacteria residing in the phyllosphere were unaffected by Shiva-1 produced in transgenic plants. Therefore, our intention is to combine engineered tolerance with the use of beneficial bacteria to further reduce the incidence of bacterial blight in Anthurium fields.

**DENDROBIUM FLOWER COLOUR**

In commercial Dendrobium hybrids, colour is predominantly determined by anthocyanins and carotenoids. Blue, red and orange colours are noticeably absent, yet are highly desired by the growers and consumers (Kuehnle et al., 2003). Chemical and molecular analyses were conducted to understand the molecular basis of flower colour in Dendrobium.

**Chemical Analysis of Pigments**

TLC and HPLC were used to separate and quantify the anthocyanidins found in Dendrobium flowers (Kuehnle et al., 1997). Pigment composition of a typical lavender cyanidin line Den. Jaquelyn Thomas ‘Uniwai Prince’ (UH503) and a rare pelargonidin line, Den. Icy Pink ‘Sakura’ (K1224) are shown in Table 2.

**Molecular Analysis of UH 503 and K1224**

We isolated a full cDNA clone encoding a key anthocyanin biosynthetic enzyme, dihydroflavonol 4-reductase (DFR) (Mudalige et al., 2004; in press), and a partial clone of putative flavonoid 3’-hydroxylase (F3’H) from Dendrobium flower buds (Mudalige, 2003). The nucleotide sequences of dfr are identical in the lavender Dendrobium Jaquelyn Thomas ‘Uniwai Prince’ (UH503) and Dendrobium Icy Pink ‘Sakura’ (K1224).

In Petunia lack of orange colour is due to the substrate specificity of the DFR enzyme. Petunia DFR does not accept dihydrokaempferol (DHK) that produces the orange pelargonidin. However, since the dfr sequences are identical in Dendrobium UH503 and K1224, substrate specificity has to be ruled out as the sole reason for the colour difference in these two Dendrobium flowers (Mudalige et al., 2004; in press). This agrees with the results from another orchid, Cymbidium. The Cymbidium DFR, which has 82 % identity and 90 % similarity (at the amino acid level) to the Dendrobium DFR, does not efficiently reduce dihydrokaempferol compared to dihydroquercetin (Johnson et al., 1999).

The lavender cyanidin producing enzyme, F3’H, is expressed in very low or negligible levels in buds and flowers in the pale orange line, K1224. This is seen by RNA blot hybridisation of buds, flowers, and leaves of the cyanidin line UH503 and the pelargonidin line K1224, using a F3’H partial clone (Fig. 7). Chemical analysis also showed kaempferol derivatives as the major flavonoid accumulated in K1224 and very low detectable levels of quercetin derivatives (Kuehnle et al., 1997). In the absence of active F3’H in K1224, our results suggest that the Dendrobium DFR enzyme can accept dihydrokaempferol, the less preferred substrate, to produce orange pelargonidin.
Blue Orchids?

We have isolated a partial cDNA clone of flavonoid 3’, 5’-hydroxylase, the enzyme responsible for the production of blue coloured delphinidin pigment. However, its expression is below detectable levels in a northern analysis containing 20 µg of total RNA from flower buds of UH503 and K1224. The presence of 3’, 5’ hydroxylated flavonols, myricetin and syringetin, suggests some activity of flavonoid 3’, 5’-hydroxylase in Dendrobium (Kuehnle et al., 1997). The petal pH was found to be ~4.79 and 5.09 for UH503 and K1224 plants. A pH value higher than 5 is suggested to be required for the blue colour in flowers (Fukui et al., 2003).

CONCLUDING REMARKS: DENDROBIUM

Genome breeding has produced many commercial Dendrobium hybrids with a variety of flower colours. Lavender cyanidin and its derivatives are the predominant anthocyanins in Dendrobium. Colours of true blue, red and orange produced by delphinidin and pelargonidin pigments are rare or lacking among commercial hybrids of Dendrobium. Molecular analysis of anthocyanin biosynthetic pathway suggests that the absence or low levels of F3’H mRNA (and hence enzyme activity) along with the acceptance of the less preferred substrate, dihydrokaempferol, by DFR might be the reason for accumulation of rare orange pelargonidin in K1224. Based on our studies, molecular breeding can increase the repertoire of flower colours in commercial Dendrobium hybrids. Introduction of a foreign DFR gene that readily accepts DFR is a sound strategy for increased intensity of orange shades in Dendrobium, while introduction of a F3’5’H gene into a mutant phenotype with low/or no F3’H activity and high vacuolar pH in flowers is a possible strategy to introduce blue shades into Dendrobium. Currently, we are in the process of determining the substrate specificity of the Dendrobium DFR in vitro to develop strategies to manipulate flower colour with the native Dendrobium genes in addition to the use of foreign genes for novel colours.

ACKNOWLEDGEMENTS

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Literature Cited


Tables

Table 1. Shiva-1 peptide concentration in transgenic lines of two Anthurium hybrids, 'Tropic Flame' and 'Paradise Pink'.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>‘Tropic Flame’</th>
<th>‘Paradise Pink’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line</td>
<td>1-1</td>
<td>1-9</td>
</tr>
<tr>
<td>Shiva-1, µM</td>
<td>0.19</td>
<td>0.38</td>
</tr>
<tr>
<td>Adjusted value, µM</td>
<td>9.5</td>
<td>19.0</td>
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Table 2. Anthocyanidin composition of lavender ‘Uniwai Prince’ (UH503) and pale orange Icy Pink ‘Sakura’ (K1224) Dendrobium orchid flowers.

<table>
<thead>
<tr>
<th>Anthocyanidin</th>
<th>‘Uniwai Prince’ (%)</th>
<th>Icy Pink ‘Sakura’ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>99</td>
<td>2</td>
</tr>
<tr>
<td>Peonidin</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Figures

Fig. 1. Agarose gel of RT-PCR products amplified from total RNA from leaves of 6 different transgenic *Anthurium* ‘Paradise Pink’ lines showing the presence of Shiva-1 transcripts. Cont is control (non-transgenic) ‘Paradise Pink’.

Fig. 2. Transgenic and wild type *Anthurium* plants were spray inoculated with a bacterial suspension of a bioluminescent strain of bacterium *Xanthomonas campestris* pv. *dieffenbachiae* (*Xcd*) at $10^5$ cfu/mL (A). X-ray film enclosed by a strip of folded construction paper was attached to the youngest fully opened leaf to monitor the internal progression of leaf infection (B). Infected area was quantified by determining the exposed area of X-ray film (C), compared to the standard guide (D).
Fig. 3. Disease progression in *Anthurium* ‘Paradise Pink’ transgenic and non-transgenic plants measured by the leaf area infected in the weeks following plant inoculation with bioluminescent *Xanthomonas campestris pv. dieffenbachiae* (*Xcd*) bacteria.

Fig. 4. Disease progression in *Anthurium* ‘Tropic Flame’ transgenic lines and non-transgenic control measured by the leaf area infected in the weeks following plant inoculation with bioluminescent *Xanthomonas campestris pv. dieffenbachiae* (*Xcd*) bacteria.
**‘Tropic Flame’**

<table>
<thead>
<tr>
<th>con</th>
<th>1-1</th>
<th>1-9</th>
<th>1-16</th>
</tr>
</thead>
</table>

Fig. 5. Northern blot showing transcript levels of Shiva-1 transgene in control (con) and transgenic lines of *Anthurium* ‘Tropic Flame’. The total RNA amount loaded in the Tropic Flame line 1-16 sample was significantly more than for either Tropic Flame 1-1 or line 1-9.

Fig. 6. Transgenic and non-transgenic control *Anthurium* plants were spray-inoculated with four beneficial bacterial species. Plants were bagged overnight and guttation fluid (E) was collected in the morning. A dilution series of guttation fluids was made and plated onto selective media (F) to count the beneficial bacterial colonies.

**UH503 K1224**

| B | F | L | B | F | L |

Fig. 7. Northern blot analysis of buds (B), flowers (F), and leaves (L) of the cyanidin-accumulating line UH503 and the pelargonidin-accumulating line K1224. RNA loading levels are shown below the blot by ethidium bromide staining of an agarose gel prior to blotting. UH503 shows putative F3’H transcripts while K1224 shows very low levels of F3’H transcripts in K1224 flowers.