

## REVIEW ARTICLE

## Temporal and spatial control of gene expression in horticultural crops

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Biotechnology provides plant breeders an additional tool to improve various traits desired by growers and consumers of horticultural crops. It also provides genetic solutions to major problems affecting horticultural crops and can be a means for rapid improvement of a cultivar. With the availability of a number of horticultural genome sequences, it has become relatively easier to utilize these resources to identify DNA sequences for both basic and applied research. Promoters play a key role in plant gene expression and the regulation of gene expression. In recent years, rapid progress has been made on the isolation and evaluation of plant-derived promoters and their use in horticultural crops, as more and more species become amenable to genetic transformation. Our understanding of the tools and techniques of horticultural plant biotechnology has now evolved from a discovery phase to an implementation phase. The availability of a large number of promoters derived from horticultural plants opens up the field for utilization of native sequences and improving crops using precision breeding. In this review, we look at the temporal and spatial control of gene expression in horticultural crops and the usage of a variety of promoters either isolated from horticultural crops or used in horticultural crop improvement.

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## INTRODUCTION

Gene expression in prokaryotes as well as in eukaryotes is regulated quantitatively and qualitatively by specific upstream DNA sequences.<sup>1</sup> These DNA sequences are commonly known as gene promoters. Initiation of transcription is in turn mediated by proteins that recognize specific DNA sequences in the promoter, thereby inducing RNA polymerase activity.<sup>2,3</sup> Promoters regulate gene expression through DNA recognition sequences, which interact with basic transcription initiation complexes and numerous transcription factors.<sup>4</sup> Such DNA recognition sequences usually include a core promoter with upstream enhancer sequences located close to the structural portion of the gene.<sup>2</sup> Transcription can be activated by these enhancer sequences independent of their location, distance or orientation with respect to the genes promoters.<sup>5</sup>

Promoters in general are divided into two regions: a core promoter region and upstream regulatory regions.<sup>6</sup> The core promoter consists of a 50–100 bp sequence adjacent to the transcription initiation site and flanking sequences.<sup>7</sup> This region interacts with the general transcription machinery<sup>8</sup> and ensures the accurate initiation of transcription by RNA polymerase II.<sup>9</sup> The core promoter consists of two key genetic elements: the TATA box (present in most promoters) and/or an initiator (Inr) element overlapping the transcription start site.<sup>10,11</sup> The initiator element binds trans-acting factors for the placement of the start site<sup>12–14</sup> and can also mediate transcription initiation in some TATA-less promoters.<sup>15,16</sup> The upstream promoter regions of 1–2 kb or more contains several *cis*-regulatory elements that serve as the binding sites for gene-specific regulators.<sup>7</sup> The regulatory sequences that play a role in the qualitative specificity of gene expression have been intensely studied.<sup>17–19</sup> Several regulatory sequences present upstream of the 5' region of plant genes include multiple *cis*-regulatory elements whose distribution and presence contribute to the expression pattern of that particular gene. This interaction between the *cis*-

acting elements and the transcription factors is key in the regulation of gene expression.<sup>20</sup> The presence of several *cis*-acting elements can contribute to the complex expression profile of a particular gene<sup>2</sup> and their differential combinatorial interactions with the transcription factors result in expression of the adjacent gene to be either constitutive, induced by external factors, tissue-specific or some combination of these.<sup>21,22</sup>

The first biotech crop commercialized in the United States was a horticultural commodity: the Flavr Savr tomato, which was submitted for approval in 1992 and released for consumption in 1994.<sup>23–25</sup> Numerous horticultural crops in the last 20 years have since been transformed with a wide range of genes and promoter elements. In most studies the introduced genes are controlled by constitutive promoters—the most popular being the 35S promoter obtained from the *Cauliflower mosaic virus* (CaMV).<sup>26,27</sup> In many cases, constitutive gene expression may not be required, especially when this does not serve a beneficial purpose.<sup>28,29</sup> In such cases, targeted gene expression using tissue-specific or inducible promoters can often provide advantages not seen using constitutive promoters.<sup>30</sup> In recent years, there has been a boom in the availability of promoter information in many promoter databases.<sup>31–33</sup> This wealth of information enables the researcher to better understand the role of promoters and their control on plant growth and development. It also allows for the development of improved cultivars containing desirable traits.<sup>34,35</sup> In this review, we look at the different promoter elements either isolated from horticultural crops or used to genetically modify a horticultural crop for improved traits (Table 1).

## PROMOTERS USED FOR CONSTITUTIVE GENE EXPRESSION

Constitutive promoters direct gene expression uniformly in most tissues and cells at all stages of plant growth and development. Constitutive promoters confer high levels of transgene expression when transferred to plant cells. They generally consist of a core DNA

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**Table 1** Description of the promoter fragments either isolated from horticultural crops or used to genetically modify a horticultural crop for improved traits

Promoter	Origin	Crop use	References
<b>Constitutive expression</b>			
BSV	Banana streak badnavirus	Banana, Sunflower	96
CaMV 35S	Cauliflower mosaic virus	Apple, broccoli, citrus, chrysanthemum, cocoa, collard, grape, Indian Mustard, Liliium, <i>Nicotiana glutinosa</i> , papaya, peach, petunia, plum, poplar, rose, strawberry, tomato, torenia	41–44, 47–63, 65–79, 84, 85, 87
CMPS	Cestrum Yellow Leaf curling virus	Grape	95
Lhca3.St.1	Potato	Chrysanthemum	100
Mannopin synthase	Gladiolus	Gladiolus	99
RoID	<i>A. rhizogenes</i>	Gladiolus	99
Uep1	Oilpalm	Oilpalm, tobacco	98
Ubiquitin	Grape, gladiolus	Grape, gladiolus	49, 99
<b>Fruit-specific expression</b>			
ACC-oxidase	Peach, apple, tomato, banana	Tomato, banana	104–107
ADP-glucose pyrophosphorylase	Watermelon	Tomato	115
Expansin	Cherry, cucumber	Tomato, cucumber	109, 110
Cucumisn	Melon	Melon	114
C11	Citrus	Lemon	117
CsACS1G/CsACS1	Cucumber	<i>Arabidopsis</i>	111
CsExp	Cucumber	Cucumber	110
DefH9	Grape	Grape	138
DFR	Grape	Grape	124
E8	Tomato	Tomato	133
Faxy11	Strawberry	Strawberry	112
GIZEP	<i>Gentiana lutea</i>	Tomato	126
Metallothionin	Citrus, oilpalm	<i>Arabidopsis</i>	118, 119
Pac1	Yeast	Avocado	136
RoIB	<i>A. rhizogenes</i>	Tomato	134
SPS	Banana	Banana	116
SIACS4/SIEXP1	Tomato	Tomato	127
<b>Seed-specific expression</b>			
2S	Grape	Grape, tobacco	145, 147
CuMFT1	Citrus	<i>Arabidopsis</i>	171
Dc3	Carrot	<i>Arabidopsis</i>	163
HaG3-A	Sunflower	Tobacco	170
LeB4	<i>Vicia faba</i>	Tobacco	156
LegA	Pea	<i>Helianthus</i>	143
NapA	<i>Brassica napus</i>	Tobacco	150
Phas	Bean	Tobacco	140, 172
Psl	Pea	Tobacco	168
Str	<i>Catharanthus roseus</i>	Tobacco	173
USP	<i>Vicia faba</i>	Tomato	158
<b>Floral tissue-specific expression</b>			
BAN215-6	<i>Brassica campestris</i>	Tobacco	249
CHS	Bean	Petunia, tobacco	197, 198
END1	Pea	Tobacco	250
GTCHS1	<i>Gentiana triflora</i>	Petunia	210
LAT52	Tomato	<i>Lilium longiflorum</i>	246
PsTL1	<i>Pyrus serotina</i>	Tobacco	214
SK2	Potato	Potato	224
TomA108	Tomato	Tobacco	248
<b>Root-specific expression</b>			
B33	Potato	Potato	284
FaRB7	Strawberry	Tobacco	267
Glb3 5'	<i>Sesbania rostrata</i>	Lotus	273
MipB	<i>Mesembryanthemum crystallinum</i>	Tobacco	268
Npv30	Bean	Lotus	275
PsENOD12A/PsENOD12B	Pea	<i>Vicia hirsuta</i>	274
RB7	Tobacco	Tomato	267
SLREO	Tomato	Tomato	263
VfLb29	<i>Vicia faba</i>	<i>Vicia faba</i>	271
Sporamin	Sweet potato	Potato, tobacco	287, 288
<b>Vascular tissue-specific expression</b>			

Table 1 Continued

Promoter	Origin	Crop use	References
AtSUC2	<i>Arabidopsis</i>	Citrus, pear, strawberries	307, 309, 310
CoYMVP	Commelina Yellow Mottle Virus	Apple	313
CsPP2	Citrus	Sweet orange	308
CsSUS1p	Citrus	<i>Arabidopsis</i> /tobacco	311
GRP 1.8	Bean	Tobacco	300
GS3A	Pea	Alfalfa	305
PAL2	Bean	Tobacco	302
RoIC	<i>A. rhizogenes</i>	Apple	313
RTBV	Rice Tungro Virus	Citrus	307
Rice sucrose synthase I	Rice	Citrus	307
<b>Inducible expression</b>			
4CL	Parsley/tobacco	Parsley/tobacco	352
CM-ACO1	Melon	Tobacco	351
HSP	Tomato	Sunflower	344
Lehsp23.8	Tomato	Tomato	341
PinII	Potato	Alfalfa, <i>Nicotiana plumbaginifolia</i> , rice	334–336
PR-1	Tobacco	Broccoli	353
PR 10	Alfalfa	Grape	354
Prosystemin	Tomato	Tomato	323
Rd29A	<i>Arabidopsis</i>	Potato	350
SWPA2	Sweet potato	Tobacco	349
Wun1	Potato	Tobacco	340

sequence (core promoter) along with other regulatory elements such as enhancers, silencers and other DNA sequences, which interact with DNA binding proteins (transcription factors) to drive transgene expression in various plant cells.<sup>27</sup> Constitutive promoters may provide ectopic gene expression in transgenic plants, not otherwise observed under normal conditions. Significantly variable results may be observed from the use of a constitutive promoter in a monocotyledonous and dicotyledonous species, which makes it essential to identify candidate promoters for specific groups to ensure high transgene expression levels.<sup>36</sup> Most constitutive promoters used for production of transgenic plants derive their origin from viral sequences. Advances in plant genome sequencing initiatives and availability of public genomic databases have led to the identification of numerous plant-derived constitutive promoters, which are increasingly being used in plant transformation.

The *Cauliflower mosaic virus* 35S (CaMV 35S or simply 35S) promoter is by far the most widely used promoter in plant transformation.<sup>37</sup> The promoter is capable of conferring high gene expression levels in most cells when transferred to plants.<sup>38</sup> The 35S promoter has been extensively studied to identify key regulatory sequences that function to provide high gene expression levels.<sup>27</sup> Sequences analyses of the 35S promoter reveal the presence of several regulatory elements that are dispersed among the entire promoter length. The promoter consists of two domains A and B, which are further subdivided into several subdomains.<sup>27</sup> Deletion analyses studies identified specific *cis*-elements in these subdomains that confer expression in specific tissues of above and below-ground plant parts. Various combinations of *cis*-elements of the 35S promoter can produce gene expression patterns that are not observed with the sole use of such elements, which suggests an interaction between *cis*-elements for expression at various plant growth and developmental stages.<sup>27</sup> Although the 35S promoter is considered to direct constitutive expression, varied expression effects result from its interaction with environmental factors<sup>39</sup> and physiological state of plant development.<sup>40</sup> Gene expression by the 35S promoter also appears to be species-dependent. For instance, high GUS expression levels were observed in pollen of transgenic strawberry plants when a 35S promoter was used, but no expression could be detected in transgenic tomato or petunia plants with similar

construct configurations.<sup>41–43</sup> In other cases, transgenic chrysanthemum expressing a GUS gene under the control of the 35S promoter exhibited weak transgene expression levels.<sup>44</sup>

In-depth functional analyses of regulatory elements present in the 35S promoter has increased our understanding of the role of individual *cis* and enhancer elements in driving gene transcription.<sup>27,45</sup> Such information has been exploited to produce chimeric versions of the 35S promoter that contain duplicated *cis* or enhancer elements.<sup>46</sup> Inclusion of additional viral- and plant-derived sequences in various combinations can provide additional synergy to the 35S promoter. Duplication of 35S enhancer elements in unique orientation along with the core promoter can greatly assist in driving high levels of several genes in a single transformation cassette.<sup>47</sup>

Genetic constructs containing a 35S-derived core promoter and either single or duplicated enhancer elements that controlled fusion gene expression, were arranged in a unidirectional (tandem) or bidirectional (divergent) orientation. Significantly high levels of GFP and GUS expression was observed in grapevine somatic embryos and plants transformed with constructs containing a bidirectional duplex promoter complex, where core promoters and duplicated enhancer elements were arranged in a divergent orientation. This phenomenon was attributed to synergistic activity of core promoters and enhancers arranged in a unique orientation.<sup>48</sup> Similar results were obtained when a grapevine *MybA1* transcription factor encoding anthocyanin expression was fused to viral promoters in various arrangements.<sup>49</sup>

The 35S promoter has been extensively used in horticultural crops for improving abiotic and biotic stress tolerance and quality traits, and for modification of plant architecture. Transgenic papaya that expressed a viral coat protein gene driven by a 35S promoter exhibited enhanced resistance against papaya ring spot virus resistance.<sup>50</sup> Following extensive field trials to confirm stability of resistance, the transgenic lines were used in breeding programs to produce virus resistant cultivars, which were deregulated and released for commercial production.<sup>51</sup> Transgenic 'Honey Sweet' plums expressing a plum pox virus coat protein under the control of the 35S promoter exhibited enhanced resistance to plum pox virus, the causal agent of Sharka disease of plum.<sup>52,53</sup> 'Honey Sweet'

was cleared for commercial production in the United States in 2010 following extensive studies by appropriate regulatory agencies.<sup>54</sup> Similar strategies have been used to incorporate virus resistance in other fruit and vegetable crops.<sup>55,56</sup>

The 35S promoter has also been fused to a number of genes coding for antimicrobial proteins to improve fungal and bacterial resistance. Improved scab resistance was demonstrated in transgenic apple that expressed a *mbr4* gene driven by the 35S promoter.<sup>57</sup> Genetically, engineered cacao plants constitutively expressing a chitinase gene showed decreased growth of *Colletotrichum gleosporioides* and reduced symptoms of necrosis compared to the controls.<sup>58</sup>

Transgenic *Citrus* plants expressing an antimicrobial peptide under control of a double-enhanced 35S promoter exhibited reduced symptoms of *Citrus* scab in greenhouse trials.<sup>59</sup> Similar results were observed in transgenic strawberries expressing an antimicrobial protein.<sup>60</sup> Transgenic grapevines expressing either antifungal or antibacterial genes under control of the 35S promoter exhibited enhanced disease resistance and are currently in advanced stages of field testing.<sup>61</sup> A number of PR proteins under the control of the 35S promoter have also been employed to engineer disease resistance in ornamentals. Delayed symptoms of fungal diseases was observed in transgenic lines compared to the controls.<sup>62</sup> Transgenic roses constitutively expressing an antimicrobial peptide exhibited resistance to powdery mildew in greenhouse trials.<sup>63</sup> In other studies insect resistant transgenic fruits and vegetables have been produced by expressing a wide array of genes driven by the 35S promoter.<sup>64-67</sup>

Transgenic horticultural crops with abiotic stress tolerance have been developed by constitutively expressing drought, cold and salinity-related genes. The *Arabidopsis* CBF transcription factors and its homologues from several species have been transferred to a number of fruit and vegetable crops for improving cold/chilling and drought tolerance.<sup>68-72</sup> A number of antiporter and vacuolar genes have been utilized for enhancing salinity tolerance in several plant species.<sup>73-75</sup>

The 35S promoter has been frequently used to downregulate genes involved in ethylene biosynthesis or fruit ripening and subsequently enhance shelf life and fruit quality.<sup>76-78</sup> Transgenic tomatoes expressing antisense versions of genes responsible for fruit softening under control of a 35S promoter exhibited enhanced shelf life due to their ability to inhibit or reduce fruit-specific enzymes responsible for softening of the fruit during the ripening process.<sup>79-81</sup> Suppression of ripening-specific *N*-glycoprotein modifying enzymes in tomato resulted in an increase in fruit shelf life without adversely affecting other qualitative characteristics.<sup>82</sup>

The 35S promoter has also been used in a number of ornamental crops to modify plant structure, flower color and engineer floral scent in flowers that normally do not produce any fragrance. Enhanced anthocyanin production was observed in transgenic tobacco and petunia plants when a maize leaf color transcription factor was constitutively expressed by a 35S promoter.<sup>83,84</sup> Transgenic flower crops with unique colorations not generally observed in wild populations have been created by isolating genes from the pigment biosynthesis pathway and placing them under control of the 35S promoter.<sup>85,86</sup> Transgenic roses and carnations expressing unique colorations were also produced and released for commercial production. Transgenic petunia with reduced height and enhanced lateral branching were produced by constitutively expressing a zinc finger transcription factor.<sup>87</sup> The enhanced branching patterns were attributed to alterations in cytokinin metabolism and increase in specific forms of cytokinins. Flowers with improved shelf life have also been produced by expressing various genes under the control of the 35S promoter.<sup>88</sup> Other efforts to improve traits in ornamental plants include the production of dwarf and compact plants and enhanced leaf color.<sup>89</sup> Several attempts to introduce floral scent have been made using genetic engineering;

such efforts have achieved partial success, mainly in part due to the absence of key enzymes or precursors that are required for the biosynthesis of the final biochemical compound.<sup>90</sup>

Chimeric promoters that drive constitutive gene expression are created by combining elements from viral-derived sequences other than the 35S promoter.<sup>91,92</sup> The Cassava vein mosaic virus (CVMV), figwort mosaic virus and Cestrum Yellow Leaf Curling Virus (CMPS) have been used to identify regulatory elements that would drive high levels of constitutive gene expression in plants.<sup>48,93-95</sup> Such chimeric promoters created through shuffling of regulatory elements and inclusion of plant-derived or other viral-derived sequences have shown high levels of transgene expression in several plant species. In some cases, the activity of viral-derived constitutive promoters has been less effective in monocotyledonous species compared to dicotyledonous plant species.<sup>94</sup> In other instances, viral-derived promoter sequences are known to direct high levels of gene expression in a wide array of dicot and monocot species.<sup>96</sup>

Advances in genome sequencing of major crops of commercial importance and availability of high throughput sequence analyses have led to the isolation of several constitutive promoters from plant species. Promoters of constitutively expressed genes such as ubiquitin are ideal candidates due to their ability to drive high gene expression levels in transformed cells. Several grapevine promoters have been isolated from the sequenced genome and analyzed for their ability to direct gene expression in various plant tissues.<sup>97</sup> Among the various candidates tested, ubiquitin promoters exhibited the highest activity levels when tested in grape somatic embryos and tobacco callus cultures, leaves and floral tissues. Two promoters Ubi-6-1 and Ubi7-2 exhibited gene expression levels comparable to a doubly enhanced 35S promoter when fused to the *gus* and anthocyanin reporter genes. Higher levels of gene expression could be correlated with an increased number of *cis*-elements in these promoters, which underlines the significance of identifying specific sequences in promoter regions for predicting expression levels. An ubiquitin extension promoter (*uep1*) identified in oil palm exhibited constitutive expression in the native species as well as in tobacco, thereby indicating its utility in monocot and dicot groups of plants.<sup>98</sup> A comparison of the activity of plant- and viral-derived promoter sequences in transgenic *Gladiolus* found no differences in expression levels of GUS during the culture stage.<sup>99</sup> However greenhouse-grown transgenic lines exhibited higher gene expression levels when the *gus* gene was driven by an *Arabidopsis*-derived *rolD* promoter. Transgenic chrysanthemums exhibited higher GUS expression levels when fused to a potato *Lhca3.St.1* promoter than the 35S promoter.<sup>100</sup> Such effects were attributed to potential post-transcriptional modifications leading to greater stability of the mRNA and higher expression levels.

## PROMOTERS INVOLVED IN FRUIT-SPECIFIC GENE EXPRESSION

The ability of constitutive promoters to direct high levels of transgene expression can be a limiting factor when temporal and spatial gene expression patterns are required to achieve manipulation of specific plant organs or developmental stages. Constitutive expression of transcription factors by the 35S promoter may interfere with normal plant development resulting in abnormal phenotypes.<sup>70,101</sup> In other cases, the 35S promoter may not be active in specific plant tissues thereby rendering it ineffective for directing high levels of spatial transgene expression.<sup>43</sup> Tissue-specific promoters may be useful for directing transgenic expression in specific plant tissues without interfering with normal plant growth and development processes. A number of promoters involved in various stages of fruit growth, maturity and ripening have been identified and can be used as genetic engineering tools to improve fruit yield, quality and post-harvest shelf life. Fruit-specific promoters with unique positive and negative regulatory elements may function efficiently in restricting



tissue-specific expression of genes and avoiding the possibility of abnormal plant growth often observed with constitutive promoters. Fruit-specific promoters from both plant species that exhibit climacteric and non-climacteric ripening patterns have been studied.

A number of fruit-specific promoters are regulated by ethylene, which is involved in a number of developmental processes including fruit maturity, ripening and senescence. Promoters of ethylene responsive genes such as the *E4* and *E8* genes have been well studied to identify activator and suppressor elements that ensure spatial and temporal gene expression.<sup>102,103</sup> Promoters from genes such as the *ACC oxidase* and *ACO synthase* isoforms that catalyze the key steps in ethylene biosynthesis have also been analyzed in a number of plant species to identify specific *cis*-elements involved in the regulatory process.<sup>104–106</sup> Deletion analysis of a peach *ACC oxidase* promoter fused to the GUS gene revealed the presence of regulatory regions that controlled gene expression at specific stages of fruit ripening.<sup>107</sup> Longer sequences of the promoter enhanced GUS expression in transgenic tomato, which was attributed to the presence of an enhancer element. Genes involved in tomato fruit development from the immature-green to mature-green stages have been identified using large-scale microarray analysis to identify fruit-specific promoters that direct gene expression from ovary development to ripening.<sup>108</sup> Analysis of a sour cherry expansin gene and its promoter region revealed the presence of a TATA box and several CAAT boxes that are conserved among promoter sequences.<sup>109</sup> Additionally, sequences that were responsive to hormones (ethylene and gibberellins), an anaerobic responsive element, GATA boxes, pyrimidine box and other *cis*-elements that conferred tissue specificity were identified in the 5' upstream region. Such sequences were highly conserved with previously identified *cis*-elements in other plant species. Promoter deletion analysis studies confirmed specific *cis*-elements that acted as positive regulators of gene expression in fruits at various stages of development. Similar results were obtained with the analysis of a cucumber fruit-specific expansion gene, *CsExp*.<sup>110</sup> In addition to the TATA and CAAT boxes, light and hormone-responsive *cis*-elements with a high degree of homology with other similar elements in other species were identified. Genes responsible for sex expression in cucumber and expressed during fruit development were studied along with their promoter regions.<sup>111</sup> Sequence analysis for two female-specific genes revealed gene duplication except for differences in the promoter regions. No differences were observed in the proximal promoter region of the *CsACS1G* and *CsACS1* genes, which has *cis*-elements that acted as repressors of gibberellins. *In silico* analysis of the distal regions indicated the presence of auxin-responsive elements in the *CsACS1G* promoter, which could potentially confer responsiveness of this gene to specific hormonal factors and control female sex expression.<sup>111</sup>

The strawberry gene *Faxy1* coding for a fruit-specific  $\beta$ -xylosidase and potentially involved in hemicellulose degradation during fruit ripening was isolated along with its 5' flanking region.<sup>112</sup> Analysis of the promoter region revealed the presence of several hormone, light and abiotic stress-related regulatory regions in addition to the TATA box and several CAAT boxes. While abscisic acid (ABA) treatment of peduncles enhanced gene expression and protein levels, a reduction was observed with NAA, GA<sub>3</sub> and ethylene treatment thereby indicating the presence of *cis*-elements that were positively and negatively regulated by specific hormones. Light responsive *cis*-elements such as ACE, SP1 G-box and MRE sequences were identified. The promoter region also included a number of cold, drought and heat-responsive elements.

A number of promoter sequences that are involved in the expression of genes involved in biochemical changes of fruit composition during development and ripening have been studied.<sup>113–115</sup> A banana sucrose phosphate synthase (*SPS*) promoter that is involved in sucrose accumulation during ripening was analyzed to identify regulatory elements and their interaction with transcription factors.<sup>116</sup>

The presence of *cis*-elements regulated by light and hormonal interactions in addition to the TATA box and CAAT box indicated an interaction of plant hormones and environmental factors during the process of fruit ripening. In watermelon, the ADP-glucose pyrophosphorylase gene, which is involved in carbohydrate metabolism during fruit ripening, was negatively regulated in the vegetative tissues.<sup>115</sup> Removal of the *cis*-elements involved in negative regulation by fine promoter deletion analysis led to constitutive expression of the gene in leaf epidermal cells. Novel fruit-specific elements were identified in a cucumis gene that is expressed in ripe melon fruits.<sup>114</sup> Deletion analysis identified a fruit-specific enhancer element, and an I-box-like sequence, which negatively regulated cucumis biosynthesis in tissues other than the fruit. Similar elements with positive and negative regulatory functions were identified in a *Citrus* C11 promoter that was specifically expressed in juice sacs of ripening lemon fruit.<sup>117</sup> Heterologous expression of the promoter: *gus* chimeric fusion in tomato revealed GUS expression specifically in the anthers and ovaries but not in vegetative tissues.

Promoters coding for metallothionin expression have been isolated from oil palm and *Citrus*.<sup>118,119</sup> The oil palm promoter exhibited higher activity in the mesocarp tissue compared to leaf tissues. A core sequence that specified mesocarp expression while negatively regulating constitutive expression was identified in addition to specific enhancer elements that promoted expression in fruit tissues. Thus, tissue-specific expression appeared to be controlled by the combination of the positive and negative regulatory elements in the promoter region.<sup>119</sup> Analysis of the *Citrus methallothionin* gene indicated the promoter to be in the TATA-less group of plant promoters such as those involved in photosynthesis. A number of fruit-specific *cis*-elements were identified in the promoter region and their function was confirmed by heterologous expression in *Arabidopsis*.

A number of genes for pigment production in fruits during the ripening phase have been well characterized.<sup>113,120–122</sup> The *VvMybA1* transcription factor is known to bind to specific regulatory elements of genes involved in the phenylalanine pathway, thereby promoting anthocyanin biosynthesis in grape berries post-veraison. A difference in the production of red and white colored berries in various grape cultivars is attributed to the insertion of a grape retrotransposon element GRET 1, which causes lack of pigment production resulting in white colored berries.<sup>123</sup> Analysis of the grape dihydroflavonol reductase (*dfr*) gene promoter region revealed the presence of regulatory elements that conferred expression in fruits during ripening.<sup>124</sup> A transcription factor *LcMybA1* that accumulated anthocyanin in litchi pericarp was found to be upregulated by light and ABA.<sup>125</sup> Promoter analysis of the *LcMybA1* gene revealed the presence of light, hormone and abiotic stress-responsive *cis*-elements that were involved in positive and negative regulation of gene expression. A *Gentiana lutea* carotenoid-related zeaxanthin epoxidase (*GIZEP*) gene and promoter region was analyzed for its function in carotenoid biosynthesis.<sup>126</sup> Heterologous expression of a *GIZEP:gus* fusion in transgenic tomato specified carotenoid expression in flowers and ripe fruit but minimal levels in vegetative tissues, roots and immature fruit containing chloroplast. *Cis*-elements that are responsive to hormones and abiotic stress factors were identified in the promoter region and may be involved in carotenoid biosynthesis at specific developmental stages. In other studies, two fruit-specific promoters in tomato, SIACS4 and SIEXP1 contained regulatory elements that conferred gene expression specifically in seed, embryo and endosperm tissues.<sup>127</sup> Candidate promoter sequences have also been identified from other fruits that exhibit seed-specific expression in heterologous species, indicating the presence of conserved *cis*-elements.<sup>128</sup>

Fruit-specific promoters have been used to either express or downregulate transgenic proteins at specific stages of development for enhancing fruit yield and quality.<sup>129–131</sup> Transgenic tomatoes expressing miraculin, a taste modifying glycoprotein under control

of an E8 promoter accumulated uniformly high levels of the transgenic protein in ripening fruits compared to fruits expressing the protein under a 35S promoter, where protein accumulation occurred predominantly in the exocarp.<sup>132</sup> Targeted expression of a yeast *S*-adenosylmethionine decarboxylase gene under the control of a fruit-specific E8 promoter significantly increased spermine and spermidine levels in transgenic tomato fruit, resulting in enhanced shelf life and higher lycopene content.<sup>133</sup> Transgenic tomatoes expressing an *Agrobacterium rolB* gene under control of an ovary-specific promoter produced parthenocarpic fruit.<sup>134</sup> No differences in fruit morphology were observed compared to the non-transformed fruit. In other studies, tomato fruits with enhanced rot resistance and shelf life were obtained by expressing a tomato anionic peroxidase under control of a fruit-specific E8 promoter.<sup>135</sup> Transgenic avocado plants harboring a *S*-adenosine *L*-methionine hydrolyase gene under control of a fruit-specific cellulose promoter have been produced to study the potential for improving fruit shelf life.<sup>136</sup> Targeted expression of a bacterial-derived auxin biosynthesis gene under control of a ovule-specific promoter significantly enhanced fecundity of transgenic 'Silcora' and 'Thompson Seedless' grapevines by improving berry and cluster size without compromising qualitative characteristics.<sup>137</sup> Similar results of improved yield along with the production of parthenocarpic fruit were obtained in transgenic strawberry and raspberry plants.<sup>138</sup>

### PROMOTERS ACTIVE IN THE SEEDS

The expression of genes that produce seed storage proteins is highly regulated. Deletion analysis of seed-specific promoters has led to identification of proximal regions that confer seed specificity and distal regions that are responsible for modulating gene expression.<sup>139–141</sup> Many seed storage protein genes have been cloned from diverse plant species, and their promoters have been analyzed in detail to identify several *cis*- and *trans*-acting elements involved in gene regulation.<sup>139,140</sup> Although such proteins exhibit wide structural variations, their promoters have a number of common properties.<sup>142</sup> They allow the synthesis of proteins at high levels in specific tissues of the seed and at certain stages of plant development.<sup>143</sup> The tightly regulated promoters make them ideal candidates for improving seed-specific traits such as nutritional value without potentially altering existing desirable characteristics.<sup>144</sup>

The 2S albumin gene promoter from a number of horticultural species has been used to direct seed-specific gene expression.<sup>145–147</sup> DNA sequence analysis of a seed-specific 2S albumin promoter region derived from grape (*Vitis vinifera* L.) indicated that several conserved seed-specific regulatory motifs were clustered within a 0.6 kb region upstream of the transcription start site. A high level of GFP expression was observed in the cotyledonary but not hypocotyl and vegetative tissues of grape and tobacco indicating the ability of the promoter to direct seed-specific gene expression.<sup>145</sup> This promoter region contained DNA motifs with core sequences identical to that of cotyledon box (CATGCA), F1 (ACGT) motif, F2 (CACCTC) motif, F3 (CACGTC) and AGGA box that have been previously characterized in 2S albumin and related seed-specific promoters of other species.<sup>147–149</sup> Substitution mutation analysis of the napin promoter using promoter-reporter gene fusions in stable transgenic tobacco showed synergistic interactions between the B-box and RY/G *cis*-elements within these complexes. It was further determined that elements in the B-box constitute an ABA-responsive complex and the seed-specific activity of the *napA* gene promoter relies on the combinatorial interaction between the RY/G complex and the B-box ABA-responsive complex during ABA response in seed development.<sup>150</sup> The B-box is highly conserved in all 2S promoters and displays similarity to abscisic acid response elements.<sup>151</sup>

Legumin gene promoters have also been well studied in a number of plant species. In *Pisum sativum*, they are coded for by

a multigene family.<sup>152</sup> The promoter regions of *legA*, *legB* and *legC* were analyzed and were found to be identical including the TATA box and CAAT box.<sup>153</sup> Deletion analysis of the pea *legA* major seed storage protein gene identified a minimal 549 bp upstream flanking sequence that was required for seed-specific expression.<sup>154</sup> This fragment contained the *leg* box element, a 28 bp conserved sequence found in the legumin-type genes of *Vicia*, *Pisum*, *Glycine* and *Helianthus*. Larger promoter fragments significantly increased levels of seed-specific gene expression.<sup>143</sup> DNA binding assays, however, indicated that the *leg* box element is not the sole promoter determinant in legumin gene expression since the –124 bp fragment which included the *leg* box did not bind to nuclear proteins.<sup>155</sup> In addition, deletion of the *leg* box with its seed protein gene-specific CATGCATG motif has no obvious effects on expression levels. A 2.4 kb fragment containing the 5'-flanking region and the 5'-noncoding sequence of the *Vicia faba* legumin gene LeB4 was observed to mediate high level of seed-specific expression in transgenic tobacco plants. Deletion analysis revealed that a 1 kb of 5'-flanking sequence was sufficient for high-levels of expression.<sup>156</sup> Similar to that observed with the pea *legA* promoter, positive regulatory, enhancer-like *cis*-elements are present within 566 bp of the upstream sequence. However, these elements are only fully functional in conjunction with the core motif CATGCATG of the legumin box present around position –95.<sup>157</sup>

Seed specificity within the 5'-upstream region of a *Vicia faba* non-storage seed protein gene, called *usp* was mainly determined by the –68/+51 region. Deletion analysis of the promoter revealed the 0.4 kb of *usp* upstream sequence contain at least six distinct interspersed *cis*-elements including an AT-rich sequence, a G-box element and a CATGCATG motif.<sup>158</sup> The beta-phaseolin gene (*phas*), encoding the major seed storage protein of bean (*Phaseolus vulgaris*), is confined to the cotyledons of developing embryos. Promoter analysis revealed that although *cis*-elements extending 1470 bp upstream of the transcription start site can modulate gene expression, the proximal 295 bp is sufficient to drive high levels of seed-specific GUS activity.<sup>141</sup> The *cis*-regulatory CACGTG motif (G box) was identified as a major *cis*-acting regulatory element conferring spatial and temporal control of beta-phaseolin<sup>159</sup> as substitution mutation of this motif reduced promoter activity by 75%.<sup>160</sup> In addition, there are three CANNTG motifs and two AG-1-binding sites in the beta-phaseolin promoter that play a critical role in gene transcription.<sup>160</sup> Combinational interactions between multiple sequence motifs such as two upstream activating sequences UAS1 (–295 to –109) and UAS2 (–468 to –391) affected the spatial and temporal regulation of the promoter. While UAS1 was sufficient for seed-specific expression, UAS2 extended gene activity to the hypocotyl. Deletion of either of the two negative regulatory sequences, NRS1 (–391 to –295) and NRS2 (–518 to –418), resulted in premature onset of GUS expression, indicating their role in the temporal control of gene expression.<sup>161</sup>

*Dc3* is a carrot *lea* class gene expressed during embryogenesis in developing seeds and in vegetative tissues subject to drought and treatment with exogenous ABA.<sup>162</sup> The proximal promoter region (–117 to +26) is responsible for mediating the embryo-specific expression.<sup>163</sup> The *Dc3* promoter directed ABA and mannitol-inducible GUS expression in *Arabidopsis* guard cells and the two treatments were additive.<sup>164</sup> A small family of bZIP transcription factors are involved in the seed-specific and ABA-responsive expression of the *Dc3* gene. *Dc3* binds to three DNA binding proteins, DPBF-1, 2 and 3. These DPBFs are bZIP factors that have been postulated to be global regulators of seed-specific and ABA-inducible genes.<sup>165</sup> Deletion analysis of the promoter region led to the delineation of a proximal promoter region and a distal promoter region. The proximal promoter region contains *cis*-acting elements responsible for the developmental regulation of *Dc3* expression in seeds. Both distal promoter region and proximal promoter region interact with common nuclear proteins that are present in embryos and

inducible by ABA in vegetative tissues.<sup>162</sup> Following a 3-day water stress cycle, leaf GUS expression increased about 200-fold while there was a 16-fold increase in free ABA. These effects were reversed by re-watering indicating the drought inducibility of this promoter. In addition, 10  $\mu$ M ABA resulted in more than 10-fold induction within 8 h.<sup>166</sup>

Other *cis*-elements involved in seed-specific promoter expression such as a number of A/T-rich sequences and a CATGCAT/A sequence are present in the 5'-upstream regions of genes encoding concavalin A (*ConA*) and canavalin, two major seed storage proteins of *Canavalia gladiata*, the sword bean. Deletion analysis of the promoter regions of both genes revealed positive regulatory elements located in the -894/-602 and -602/-74 regions of the *ConA* gene, and in the -428/-376, -281/-155 and -155/-50 regions of the canavalin gene.<sup>167</sup>

Progressive 5' deletions of the pea lectin (*PsI*) gene promoter identified a 22 bp element (W1), important for seed-specific expression when coupled as a trimer to a heterologous TATA box.<sup>168</sup> Within the 469 bp upstream region of the seed-specific pea lectin gene, a trimer of the 22 bp fragment conferred high gene expression in seeds. This 22 bp fragment contains the binding site for the cloned basic domain/leucine zipper (bZIP) proteins TGA1a and Opaque-2 (O2), which in turn binds to the C-box *cis*-element (ATGAGTCAT).<sup>169</sup> In a majority of the promoters, most of the *cis*-elements are located within 1 kb upstream of the ATG sequence. However, in the HaG3-A sunflower promoter that directs helianthinin gene expression, *cis*-regulatory elements located in a 2.4 kb upstream region were responsible for expression in a heterologous system.<sup>170</sup> Similarly, the 2.4 kb in the 5' upstream region of the *CuMFT1* (citrus FT/TFL1 homolog from Satsuma mandarin (*Citrus unshiu* Marc.)) contained RY (CATGCAT), E-box (CANNTG) and distant B-box (GCCACTTGTC) *cis*-elements, all of which have been reported to promote seed-specific gene expression in plants. Seed-specific expression was confirmed by expressing the *gus* gene in *Arabidopsis*.<sup>171</sup> A 0.8 kb fragment from the 5'-flanking region of a French bean *beta-phaseolin* gene yielded strong, temporally regulated and embryo-specific GUS expression in transgenic tobacco plants.<sup>140</sup> Expression levels were observed to be similar as that obtained using the phaseolin seed protein promoter.<sup>172</sup>

Several promoters expressed in the seeds can also be expressed in other plant organs. The *strictosidine synthase* (*Str*) gene promoter from *Catharanthus roseus* contains a G-box sequence which helps to direct seed-specific expression independently of other regulatory sequences. G-box-directed expression in leaves, however, required the presence of an enhancer region from the 35S promoter.<sup>173</sup> The fruit and seed-specific expression of two tomato fruit-specific promoters *SIACS4* and *SIEXP1* was analyzed in transgenic tomato lines expressing the promoter: *gus* fusion constructs. The *SIACS4* promoter (-1 to -373) showed GUS activity restricted specifically to flower buds and seeds in fruits. On the contrary, the *SIEXP1* promoter (-1 to -769) showed high level of expression in seeds as compared to fruit tissues at different stages of fruit ripening.<sup>127</sup> The seed-specific expression shown by these promoters might be due to the presence of Prolamin box and E-boxes, which are conserved sequences found in the promoters of many seed storage proteins.<sup>150</sup>

## PROMOTERS ACTIVE IN THE FLORAL TISSUES

In contrast to other plant organs, flowers are composite structures composed of several organs that form an ordered pattern.<sup>174</sup> The typical flower consists of four organs arranged in whorls. The sepals consist of the outermost whorl followed by the petals in the next whorl and stamens (male reproductive organs) in the third whorl and carpels (female reproductive organs) in the innermost whorl.<sup>175</sup> Each of these whorls consist of unique genes targeted to the specific organ and several homeotic genes that affect the fate of organ

primordia.<sup>176</sup> Targeted genetic engineering, by utilizing promoters obtained from genes specifically expressed in a specific whorl is highly desirable for targeted gene expression and can be exploited by using specific promoters.<sup>177</sup> Some of the traits that can be engineered in the floral tissues include increased vase life,<sup>178-182</sup> flower color modification,<sup>181,183-185</sup> fragrance<sup>185-187</sup> and male and female sterility<sup>188-192</sup> among others.

Chalcone synthase (*CHS*) is synthesized in the flower corolla, tube and anthers<sup>193</sup> and is important for the biosynthesis of flavonoid antimicrobial phytoalexins and anthocyanin pigments in plants.<sup>194</sup> Various *CHS* promoters has been studied extensively in many plants, especially in *Phaseolus vulgaris*, antirrhinum, petunia and parsley.<sup>195-197</sup> A 1.4 kb promoter fragment of the bean *CHS8* gene was highly active in the root apical meristem and in petals and weakly expressed in other floral organs, mature leaves, and stems.<sup>198</sup> Gene expression strongly depended on the G-box and H-box,<sup>199</sup> as a synthetic 39 bp DNA fragment containing the two elements and linked to the minimal cauliflower mosaic virus 35S promoter conferred a high level of tissue-specific expression. Mutations in either the G-box or H-box motifs abolished tissue-specific gene expression.<sup>195</sup> A mutation in the G-box did not exhibit impaired promoter response to wounding, but demonstrated a 19% reduction in the response to HgCl<sub>2</sub> and TMV. A mutation at the H-box resulted in a 30% increase in promoter response to wounding and reductions of 36% and 54% in the response to HgCl<sub>2</sub> and TMV, respectively, demonstrating the differential utilization of regulatory *cis*-elements.<sup>200</sup> A silencer element present between positions -140 and -326 contained three binding sites for a bean nuclear factor (SBF-1).<sup>201</sup> The region between -326 and -130 contained both activator and silencer elements.<sup>202</sup> The petunia genome contains eight chalcone synthase genes, of which four are differentially expressed in floral tissues and UV light-induced seedlings.<sup>197</sup> The *chsA* promoter contains a 220 bp *cis*-acting element region conferring flower-specific and UV-inducible expression<sup>203</sup> and its expression was enhanced when plant tissues were exposed to high carbohydrate levels.<sup>204</sup> A promoter fragment from -67 to +1, was able to direct low level flower-specific gene expression, but could not drive UV-inducible expression in transgenic *Petunia* seedlings.<sup>205</sup> Histochemical analyses of GUS expression demonstrated that *CHS* promoters are not only active in pigmented cell types (epidermal cells of the flower corolla and tube and subepidermal cells of the flower stem), but also in a number of non-pigmented cell types (mesophyll cells of the corolla, several cell types in the ovary and the seed coat).<sup>197</sup> The highest level of expression directed by the 1.1 kb snapdragon chalcone synthase promoter was observed in immature seeds. Deletions analysis identified regions of the promoter required for expression in roots, stems, leaves, seeds and flower petals of transgenic plants. A promoter fragment truncated to -39 activates transcription in roots of 4-week-old seedlings, whereas a fragment extending to -197 bp directed expression in petals and seeds.<sup>206,207</sup> The positive regulatory element in the promoter consists of a 47 bp direct repeat between positions -564 and -670.<sup>208</sup> 150 bp of the 5' flanking region contained *cis*-acting signals for UV light-induced expression.<sup>209</sup> The *GTCHS1* promoter from *Gentiana triflora* contains a sequence of the MYB protein-binding site, five consensus sequences of the MYC protein-binding site, one core sequence of a G-box and three P-box-like sequences. Gene expression is strongly directed flower limbs and the inner epidermis<sup>210</sup> and is dependent on the G-box.<sup>211</sup>

In efforts to produce high transgene expression in petal tissue of ray florets of chrysanthemum, expression levels were compared with four petal-specific promoters: ubiquitin extension protein (*UEP1*) promoter from chrysanthemum chalcone synthase (*chs-A*) a zinc finger transcription factor (*EPF2-5*) from petunia, eceriferum (*CER6*) from *Arabidopsis* and multicystatin (*PMC*) from potato. The highest expression in petal tissue of ray and disc florets was



conferred by the *UEP1* promoter, followed by *CER6* and *EPF2-5*. The *UEP1* promoter in ray florets was reported to confer over 50-fold enhancement in expression as compared to CaMV 35S-based promoters.<sup>212</sup>

Promoters targeting other parts of the flower have also been evaluated. When a 2.4 kb fragment of the pistil-specific thaumatin/PR5-like protein (*PstL1*) promoter from Japanese pear (*Pyrus serotina*) was evaluated,<sup>213</sup> it was observed that *PstL1* accumulated in pistils but not in other floral and vegetative organs which constitute a novel pistil-specific class of thaumatin/PR5-like protein.<sup>214,215</sup> Other parts of the flower targeted include the flower receptacle. Promoters targeting other parts of the flower have been evaluated. When a 2.4 kb fragment of the pistil-specific thaumatin/PR5-like protein (*PstL1*) promoter from Japanese pear (*Pyrus serotina*) was evaluated,<sup>213</sup> it was observed that *PstL1* accumulated in pistils, but not in other floral and vegetative organs which constitute a novel pistil-specific class of thaumatin/PR5-like protein.<sup>214,215</sup> Several reports exist on the isolation, characterization and use of promoters targeted to the flower receptacles,<sup>216,217</sup> stamen,<sup>218–220</sup> anthers<sup>221–223</sup> and ovaries.<sup>134</sup> The potato *SK2* gene promoter directed pistil-specific gene expression. It was observed that the regulatory elements responsible for pistil-specific expression were located within a 230 bp fragment.<sup>224</sup>

Numerous genes and their promoters that are expressed at the various stages during male gametogenesis have been cloned.<sup>225</sup> Most of these have been isolated from agronomic crops such as maize,<sup>226,227</sup> rice,<sup>228,229</sup> tobacco<sup>230,231</sup> and wheat<sup>232</sup> as well as the model plant *Arabidopsis*.<sup>233–236</sup> A few have also been isolated from horticultural crops.<sup>237</sup> These promoters fused to a cytotoxic gene have been used to induce male sterility.<sup>226,238</sup> The *LAT52* and *LAT59* anther-specific gene promoters from tomato have been evaluated in various crops for their anther-specific activity.<sup>237,239,240</sup> These genes are very critical during tomato pollen development. In their absence, pollen germinates abnormally and is sterile.<sup>241</sup> All major *cis*-regulatory elements required for pollen-specific transcription in the *LAT52* promoter were located within –492 to –52.<sup>242</sup> Both promoters became active with the onset of microspore mitosis and increased progressively until anthesis,<sup>223</sup> although the *LAT52* promoter demonstrated a minor temporal difference in activity when tested in different plant species.<sup>243–245</sup> The *LAT52* promoter was highly active in electroporated pollen protoplasts isolated from *Lilium longiflorum*.<sup>246</sup> The antisense *Bcp1* gene under the control of the *LAT52* promoter induced sterility in cauliflower pollen.<sup>247</sup> Similarly, a 0.44 kb *chiA* PA2 promoter fragment from petunia drove pollen-specific gene expression and a 1.75 kb *chiB* PB promoter fragment conferred anther-specific (pollen and tapetum cells) expression to the *gus* gene.<sup>222</sup> The *TomA108* gene promoter from tomato was also highly active from early-meiosis to free microspores production in the tapetum.<sup>248</sup> Deletion analysis of the *BAN215-6* gene promoter isolated from the Chinese cabbage identified a 383 bp (–274–+109) region that was observed to be sufficient for the anther-specific expression of the *gus* gene. GUS expression was first detected in uninucleate microspores, increased during anther development and reached its highest level in mature pollens.<sup>249</sup> Similar observation were made with the 2.7 kb promoter fragment of a pea *END1* gene. This promoter was evaluated in several species and observed to be fully functional in the anthers.<sup>250</sup>

## PROMOTERS ACTIVE IN THE ROOT SYSTEM

Plant roots have been essential for the evolution of vascular plants enabling them to meet the requirements for anchorage and the acquisition of water and nutrients.<sup>251</sup> Roots are multifunctional and involved in the acquisition of water and nutrients, anchorage of the plant and storage functions.<sup>252,253</sup> In fact, plant productivity is dependent on a healthy root system<sup>254</sup> as problem with root health directly affects the above ground part.<sup>255,256</sup> Roots interact with its surrounding environment<sup>257</sup> and can be susceptible to a multitude

of problems stemming from the environment in which it lives.<sup>252,258–260</sup> Targeted gene expression by using root-specific promoters can allow for the development of horticultural plants better suited for growth in a range of soil types, soil pH and under microbial stress.<sup>261,262</sup> Several root-specific promoters have been evaluated in horticultural plants. The *SLREO* gene isolated from tomato is highly expressed in roots, but had a very low level of expression in aerial plant organs.<sup>263</sup> The RB7 protein from tobacco,<sup>262</sup> is a membrane channel aquaporin, allowing the diffusion of amino acids and/or peptides from the vacuolar compartment to the cytoplasm.<sup>264,265</sup> This promoter is root-specific and has been used to drive the *Arabidopsis* thionin (*Thi2.1*) gene in tomato.<sup>266</sup> A strawberry homolog (*FaRB7*) behaves in the same way as the tobacco RB7 promoter.<sup>267</sup> Other promoters identified include a 2 kb promoter fragment of the *MipB* gene from *Mesembryanthemum crystallinum* that was observed to be expressed strongly in the tobacco root. However, gene expression was also observed in other rapidly expanding cells and cells with high water flux capacity.<sup>268</sup>

Several root nodule-specific promoters have been identified from leguminous plants.<sup>269</sup> A 1.3 kb fragment of the French bean *gln-gamma* gene promoter is strongly induced during nodule development.<sup>270</sup> The *Vicia faba* *Vflb29* gene promoter was found to be specifically active not only in the infected cells of the nitrogen-fixing zone of root nodules but also in arbuscule-containing cells of transgenic *V. faba* roots colonized by the endomycorrhizal fungus *Glomus intraradices*.<sup>271</sup> A promoter fragment (–692/41) encoding the *Vicia faba* early nodulin *VfEnod12* and containing a putative binding site for the transcription factor ENBP1, mediated reporter gene expression in root cortical cells, nodule primordia and the prefixing zone II of transgenic *Vicia hirsute* root nodules.<sup>272</sup> A 1.9 kb fragment of the *Sesbania rostrata* leghemoglobin *glb3* 5'-upstream region was found to direct a high level of nodule-specific GUS activity in lotus. Replacement of the –161 to –48 region, containing the *glb3* CAAT and TATA boxes, with the heterologous truncated promoters delta-p35S and delta-pnos, resulted in a loss of nodule specificity and reduction of GUS activity restricted to the *Rhizobium*-infected cells of the nodules.<sup>273</sup> Promoter analyses of pea *PsENOD12A* and *PsENOD12B*, nodulin gene promoters showed that the 200 bp immediately upstream of the transcription start are sufficient to direct nodule-specific and Nod factor-induced gene expression.<sup>274</sup> GUS activity was only detected in the infected cells of the nodules of lotus transgenic plants when a *Npv30* promoter isolated from *Phaseolus vulgaris* fused to the *gus* reporter gene was used.<sup>275</sup>

Several genes are highly upregulated in tubers.<sup>276–278</sup> Many of these storage gene promoters have been exploited for horticultural crop improvement. Patatin is a major tuber protein and is very tissue-specific.<sup>279</sup> The 1.5 kb 5'-upstream region of the class I patatin gene *B33* directed strong expression of the GUS reporter gene in potato tubers which was on average 100- to 1000-fold higher in tubers as compared to leaf, stem and roots.<sup>280</sup> Gene expression was also induced by sucrose application.<sup>278</sup> Deletion analysis identified a tuber-specific element located downstream from position –195. Sequences between –40 and –400 bp and between –400 and –957 bp of the transcriptional start site were able to confer tuber-specific expression on a heterologous truncated promoter.<sup>281</sup> Sucrose inducibility was controlled by sequences downstream of position –228.<sup>282,283</sup> High levels of mature human serum albumin was expressed in potato tubers using the potato patatin *B33* tuber-specific promoter.<sup>284</sup>

Sporamin accounts for more than 80% of the total soluble proteins of tuberous roots of sweet potato<sup>285</sup> and can be induced by wounding and sucrose.<sup>286</sup> Two wound response-like elements, a G box-like element and a GCC core-like sequence, were found in the sporamin gene promoter.<sup>287</sup> When overexpressed in potato, the sporamin promoter was highly active in leaves, stems and different size tubers.<sup>288</sup> Deletion of the sporamin A promoter sequences extending from position –305 (relative to the transcription start



site) to -283 and from -146 to -87 resulted in an approximately 40-fold enhancement in GUS reporter expression. It was observed that the sequence between positions -282 and -165 contained to two *cis*-acting elements, termed CMSREs (carbohydrate metabolite signal responsive elements) 1 and 2 are responsible for the sucrose-responsiveness of the promoter.<sup>289</sup>

### PROMOTERS ACTIVE IN THE VASCULAR TISSUES

The plant's vascular system acts as a bridge between the leaves and other parts of the shoot, with the roots.<sup>290</sup> This system, comprised of two kinds of conducting tissue, the xylem and phloem enables efficient long-distance transport between the organs.<sup>291</sup> Xylem is primarily responsible for water transport and movement of soluble mineral nutrients from the roots throughout the plant.<sup>292</sup> Phloem, on the other hand, transports sugars from source tissues such as the photosynthetic leaf cells to sink or storage tissues such as the roots, flowers or fruits.<sup>293,294</sup> Targeting a transgene into the vasculature using either a xylem or phloem-specific promoter allows gene expression at the site of infection and can potentially control vascular maladies. It can also provide a rapid response in response to wounding and for the control of aphids and other sap sucking insects.<sup>295-297</sup>

A 494 bp promoter fragment of the glycine-rich wall protein GRP 1.8 from the French bean translationally fused to the *gus* gene expressed the gene in vascular tissue of roots, stems, leaves and flowers. Four *cis*-acting regulatory regions, SE1 and SE2 (stem elements), a negative regulatory element and a root-specific element, were found to control the tissue-specific expression.<sup>298</sup> The vs-1 motif in the GRP 1.8 promoter was a *cis*-element that specifically bound to a transcription activation factor VSF-1 protein and allows xylem-specific expression.<sup>299</sup> The gene was developmentally expressed during differentiation of both primary and secondary vascular tissue and was also rapidly induced (within <30 min) after excision-wounding of young stems.<sup>300</sup> The bean phenylalanine ammonia-lyase gene 2 (*PAL2*) is expressed in the early stages of vascular development at the inception of xylem differentiation. Deletion analysis revealed the presence of *cis*-elements located between nucleotides -289 and -74 relative to the transcription start site being essential for xylem expression.<sup>301</sup> Expression of the *PAL2* promoter in the vascular system involves positive and negative regulatory *cis*-elements. Among these elements is an AC-rich motif implicated in xylem expression.<sup>302</sup> Similarly, the citrus *PAL* gene (*CsPP*) promoter fused to the *gus* gene and transformed into tobacco and 'Valencia' sweet orange preferentially, but not exclusively, conferred gene expression in xylem tissues of tobacco. Weaker GUS staining was also detected throughout the petiole region in tobacco and citrus *CsPP* transgenic plants.<sup>303</sup> The *Arabidopsis* *PAL* promoter when transformed into citrus expressed exclusively in the xylem parenchyma.<sup>304</sup>

The full-length promoter and a series of 5' deletions of the pea cytosolic glutamine synthetase *GS3A* gene were fused to the *gus* gene and introduced into tobacco and alfalfa. The *GS3A* promoter directed GUS expression in the phloem cells of the vasculature in leaves, stems and roots. Interestingly, the promoter was found to be active even when deleted to -132 relative to the start of transcription.<sup>305</sup> The *Arabidopsis* sucrose-H<sup>+</sup> symporter *AtSUC2*<sup>306</sup> has been used to direct phloem-specific gene expression in a number of horticultural crops, such as Mexican lime,<sup>307</sup> sweet orange,<sup>308</sup> pears<sup>309</sup> and strawberries.<sup>310</sup> Two alleles of the *Citrus sinensis* sucrose synthase-1 promoter (*CsSUS1p*) were inserted upstream of the *gus* gene to test their ability to drive expression in the phloem of transgenic *A. thaliana* and *N. tabacum*. Although both promoter variants were capable of conferring localized GUS expression in the phloem, the *CsSUS1p-2* allele also generated a significant level of expression in non-target tissues. Deletion analysis of the *CsSUS1p* suggested that a fragment comprising nucleotides

-410 to -268 relative to the transcriptional start site contained elements required for phloem-specific expression, while nucleotides -268 to -103 contained elements necessary for wound-specific expression.<sup>311</sup> In citrus, the *CsSUS* promoter appeared leaky with some laminar tissue staining.<sup>312</sup> A citrus phloem protein 2 (*CsPP2*) promoter was also evaluated in sweet orange and gene was observed to be preferentially expressed in the phloem.<sup>308</sup> Two heterologous promoters, *rolC* and *CoYMVP*, were fused with the *gus* reporter gene and evaluated in the vegetative tissues of apple. It was observed that the *CoYMV* promoter was slightly more active than the *rolC* promoter, although both expressed GUS at a lower level than the *CaMV 35S* promoter. This analysis demonstrated that with both the *rolC* and *CoYMV* promoters the reporter gene activity was primarily localized to vascular tissues, particularly the phloem.<sup>313</sup>

### INDUCIBLE PROMOTERS

These promoters are induced by either physical factors such as biotic and abiotic factors or chemical agents and is a powerful tool to regulate the expression of genes at certain stages of plant or tissue development.<sup>314-318</sup> Examples of physically regulated promoters include heat shock promoters,<sup>319</sup> cold inducible promoters,<sup>320</sup> light inducible promoters,<sup>321</sup> light repressible promoters<sup>322</sup> or wound inducible promoters.<sup>323</sup> Chemically inducible promoters include alcohol regulated promoters,<sup>324</sup> tetracycline regulated promoters,<sup>325</sup> steroid responsive promoters such as glucocorticoid receptor promoters, estrogen and ecdysone receptor promoters,<sup>316,326</sup> metal-responsive promoters<sup>327</sup> and pathogenesis related promoters.<sup>328</sup> Some of these promoters have been isolated from horticultural crops or used for horticultural plant improvement.

The potato proteinase inhibitor II gene (*pinII*) is a chymotrypsin and trypsin inhibitor<sup>329</sup> and is wound and UV irradiation inducible.<sup>330,331</sup> The sequence TATAAA is found 26 nucleotides upstream of the transcription initiation site and the sequence CAAAT at position -103 in the promoter.<sup>332</sup> The wound inducibility of this promoter has been evaluated in several plant species to test gene function that involve cell-specific and systemic induction.<sup>333</sup> The *PinII* promoter has been utilized in the wound-inducible expression of the bacterial isopentenyl transferase (*ipt*) gene into *Nicotiana glauca* and *plumbaginifolia*.<sup>334</sup> In transgenic rice plants, the expression of the *pinII-gus* fusion gene displayed a systemic wound response.<sup>335</sup> In alfalfa, GUS expression was observed in leaf and root vascular tissue, and in some plants, expression was observed in leaf mesophyll cells. Mechanical wounding of leaves increased GUS expression approximately twofold over 24 h.<sup>336</sup> The *PinII* promoter is active in monocot species also. Localized induced gene expression was obtained in white spruce seedlings (*Picea glauca*) using a similar *pinII-gus* construct.<sup>337</sup> In rice, the wound-inducible expression of the *pinII* gene driven by its own promoter, together with the first intron of the rice actin 1 gene (*act1*), resulted in high-level accumulation of the *PINII* protein in the transgenic plants.<sup>338</sup> The *wun1* gene is another wound inducible gene from potato.<sup>339</sup> Histochemical analysis of transgenic tobacco plants that expressing the *wun1-gus* fusions demonstrated the wound-inducible and cell-specific *wun1* promoter activity in plants containing the -1022 bp fragment.<sup>340</sup>

The tomato *Lehsp23.8* heat shock protein gene's expression is induced by treatment with high or low temperatures, heavy metal or ABA. Using the *gus* reporter gene system, the developmental and tissue-specific expression of the *gus* gene controlled by the *Lehsp23.8* promoter was characterized in transgenic tomato plants. The optimal heat-shock temperatures leading to the maximal GUS activity in the pericarp of green, breaker, pink and red fruits were 42, 36, 39 and 39 °C, respectively.<sup>341</sup> Deletion analysis of the *Lehsp23.8* promoter revealed a proximal region (-565 to -23 bp) to harbor *cis*-regulatory elements that conferred high levels of heat-induced expression in transgenic tobacco. Mutation of the five proximal

HSEs (HSE1 to 5) led to an absence of heat inducibility.<sup>342</sup> The tomato chloroplast small heat shock protein (HSP), *HSP21*, is also induced by heat treatment in leaves.<sup>343</sup> Several sunflower genes encode small HSPs.<sup>344,345</sup> In vegetative tissues, these mRNAs accumulated in response to either heat shock (42 °C), ABA or mild water stress treatments. The *Hahsp17.7G4* mRNA is also active during zygotic embryogenesis at 25 °C. Developmental induction of the G4 promoter was faithfully reproduced during zygotic embryogenesis in transgenic plants containing G4: GUS translational fusions. Distal sequences of this promoter (between -1132 and -395) were needed to confer a preferential spatial expression of GUS activity in the cotyledons while proximal regions confer responses to ABA and heat shock<sup>346</sup> This -83 to +163 fragment was observed to be sufficient to support a promoter activity in tobacco galls induced by the root-knot nematode *Meloidogyne incognita*. GUS activity was largely restricted to giant cells within the galls.<sup>347</sup> However, the *Hahsp17.6G1* (G1) promoter which is not induced by heat shock, was observed to be silent in these giant cells, indicating that the high metabolic rate of giant cells produced as a result of nematode infection may somehow mimic heat-shock and/or other stress responses.<sup>348</sup> Other examples include the strong oxidative stress-inducible peroxidase *SWPA2* promoter from sweet potato. This promoter contained several *cis*-element sequences implicated in oxidative stress such as GCN-4, AP-1, HSTF and SP-1 reported in animal cells and a plant-specific G-box. A 1314 bp promoter fragment fused to the *gus* gene and transformed into tobacco exhibited about 30 times higher GUS expression than the CaMV 35S promoter in response to environmental stresses including hydrogen peroxide, wounding and UV treatment.<sup>349</sup> Similarly, when potatoes were transformed with a stress inducible *Arabidopsis* rd29A promoter driving the cold tolerance CBF genes, freezing tolerance was increased by 2 °C.<sup>350</sup>

Several promoters are chemically induced. Ethylene treatment or leaves wounding rapidly induced the melon *ACC oxidase* gene, *CM-ACO1-gus* gene in transgenic tobacco plants.<sup>351</sup> Jasmonates and alpha-linolenic acid strongly induced the expression of the wound-induced 4CL promoter in parsley cell cultures and transgenic tobacco plants expressing 4CL1-*GUS* gene fusions. This supported a role for jasmonates in mediating wound-induced gene expression.<sup>352</sup> Two wound response-like elements, a G box-like element and a GCC core-like sequence were found within a 1.25 kb *sporamin* promoter. Transgenic tobacco containing this promoter driving the *gus* gene was wounded and a high level of GUS activity was observed in stems and leaves of, but not in roots. Exogenous application of methyl jasmonate also activated the *sporamin* promoter in leaves and stems of sweet potato.<sup>287</sup> The chemically inducible PR-1a tobacco promoter was fused to the *Bacillus thuringiensis cry1Ab* gene and transformed into broccoli. Two progeny lines expressed the *cry1Ab* gene and provided insect resistance when treated with the chemical inducers 2,6-dichloroisonicotinic acid or 1,2,3-benzothiadiazole-7-carbothioic acid 5-methyl ester.<sup>353</sup> Other examples include the alfalfa pathogen-inducible PR 10 promoter. This promoter fused to the *Vitis* stilbene synthase 1 (*VvSS1*) gene was introduced into the grape rootstock genome. Transgenic plants accumulated 5- to 100-fold resveratrol in leaves infected with *Botrytis* using an *in vitro* test.<sup>354</sup>

Some promoters can be regulated both physically and chemically. A 2.2 kb promoter region of the tomato prosystemin gene fused to the *gus* gene and transformed back into tomato contains elements conferring its correct temporal and spatial expression in the vascular bundles of transgenic tomato plants by wounding and by treatment of the plants with methyl jasmonate.<sup>323</sup>

## CONCLUSIONS

The global human population is increasing at an unprecedented rate and is projected to cross 11 billion before the end of this

century.<sup>355</sup> This doubling of the population and a rapid increase in global food demand creates huge challenges for the sustainability both of food production and the ability to grow more from a shrinking cultivable land mass. Thus far, the combined effects of improved varieties, increased fertilizer use and irrigation coupled with increased pesticide use have been instrumental in allowing world food production to double in the last 35 years.<sup>356</sup> A multifaceted and linked global strategy to increase food production from shrinking land and water resources will ensure sustainable and equitable food security.<sup>357,358</sup> Fruits and vegetables claimed an increasing share of the world agricultural trade, from 10.6% in 1961 to 17% in 2001.<sup>359</sup> It is expected that demand for horticultural commodities, especially fruits, vegetables and flowers will continue to increase with the increase in the purchasing ability of the expanding middle class and an growing awareness of the many health benefits associated with an increased consumption of fruits and vegetables.<sup>360,361</sup>

Acreage under genetically modified crop plants has increased substantially in recent years as more and more acreage is consumed to feed, clothe and sustain a growing world population.<sup>362</sup> However, there has been limited progress in the commercialization of genetically modified horticultural commodities, with the exception of the Hawaiian papaya cultivars resistant to papaya ringspot virus<sup>363,364</sup> and color-altered varieties of carnation flowers.<sup>365</sup> Development of genetically modified horticultural cultivars that can alleviate consumer concerns and the related reluctance of food processors and marketers to accept new biotech horticultural commodities can speed up the introduction of horticultural products already developed.<sup>366</sup>

In recent years, molecular advancement in the field of bioinformatics has been rapid.<sup>37</sup> With the genome of a number of horticultural species being sequenced and the availability of numerous online databases for analyzing, identifying and characterizing promoters from different horticultural species,<sup>367-371</sup> it has become relatively easier to identify and characterize plant derived promoters and other genetic elements. Identification and incorporation of plant promoter and other genetic sequences by exploiting the expanding public databases and bioinformatics services can potentially alleviate some of the public concerns about safety issues with the use of a genetically modified horticultural crop.<sup>372,373</sup> Development of precision breeding techniques (previously termed as cisgenic or intragenic genetic improvement)<sup>374</sup> will enable more precise genetic modification of plants.<sup>375</sup> The resulting horticultural plant, devoid of DNA from other gene pool and restricted to a modulation of existing traits from the sexually compatible gene pool, could also result in less comprehensive regulation towards the release of a precision bred plant, thereby decreasing the regulatory approval costs.<sup>373</sup>

## COMPETING INTERESTS

The authors declare no conflict of interest.

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