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Promoters Used in Genetic Transformation of Plants

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Abstract: The promoter is a DNA sequence that regulates the expression of a particular gene. Knowledge of the promoters used in gene constructs for genetic transformation is essential for successfully applying GM technology. Promoters can be classified as constitutive, inducible and organ/tissue specific. The use of inducible and organ/tissue specific promoters has gained importance, because of its extensive applicability. This review presents a summary of the different types of promoters with examples of some of them that have been identified and characterized for genetic transformation of various plant species.

Key words: Biotechnology, inducible promoter, specific promoter, organ/tissue, applicability

INTRODUCTION

Conditions of biotic and abiotic stress in agricultural crops can cause large yield losses worldwide. However, progress in the generation of transgenic plants with increased tolerance to such stresses, has been slow. Heterogeneous conditions in the field, combined with abiotic stress and global climate change are just some of the challenges of modern agriculture (Mittler and Blumwald, 2010).

Genetic transformation opens new perspectives in breeding programs, expanding and providing new genes for certain characteristics imposed by sexual incompatibility or genetic variability (Sartoretto *et al*., 2008). The generation of genetically modified plants is a tool to achieve desirable characteristics in a breeding program highlighting the achievement of higher productivity and a lower impact on the environment. This technology also makes it possible to obtain plants that are more tolerant of different types of stress such as drought and cold and more resistant to diseases and pests. As indicated by Mittler and Blumwald (2010), a combination of innovative approaches that take into consideration the physiological and genetic basis of different cultures, the use of enzymes and proteins from other organisms and other tools of genetics and improvement will be necessary to significantly improve theplants' tolerance of external factors. Studies related to the identification and characterization of inducible and organ/tissue specific promoters are interesting to allow genetic manipulation and explore the genetic potential of several species of agronomic and forestry interest. The choice of the promoter used to construct the transgene depends mainly on the intended goals of genetic transformation (Potenza *et al*., 2004). Specific promoters may direct the expression of genes which confer resistance to pathogens in a directed manner (Twyman, 2003). In the case of toxins acting against pests, it is possible to limit gene expression to only onetarget organ of the plant, preventing the presence of toxins in the product that will be consumed by the population and also in other organs that are used in animal nutrition, thereby reducing the probability of affecting non-target organisms (Potenza *et al*., 2004). For production of biopharmaceuticals, the use of organ-specific promoters is important to express the gene of interest in those organs that are able to produce the protein in an appropriate manner (Twyman *et al*., 2003).

IMPORTANCE OF PROMOTERS

The promoter is the central processor of regulation of a gene, since it contains binding sites for RNA polymerase and general transcription factors responsible for gene transcription. Transcription factors, in turn are activated under different situations such as endogenous (auxin, gibberellin, salicylic acid, jasmonic acid) and exogenous stimuli (light, pressure, humidity, temperature). The combination of promoter and transcription factors action determines the activation or repression of gene expression (Smale and Kadonaga, 2003).

For most genes encoding proteins, transcription initiation includes binding and activation of RNA polymerase II (Potenza *et al*., 2004) this step being the

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most regulated in gene expression. It is essentially controlled by the promoter region of the gene (Singh, 1998). In eukaryotes, the promoter region in general has a conserved sequence (T/A) A (A/T) at about 30 base pairs (bp) of the transcription initiation point which is termed TATA box and elements near the promoters which are located approximately 100 (CCAAT box) and 200 bp (GC box) above the start point of transcription (Smale and Kadonaga, 2003).

Processes that provide transcriptional modulation are extremely complex; the elements contained in the promoter sequences usually determine the correct starting point of transcription, acting as activators or repressors, indicating the place and the moment that this biological process should occur (Butler and Kadonaga, 2002).

Promoters are a key tool in biotechnological processes to ensure that expression of a gene of interest is effective and regulated. The availability of promoters that differ in their ability to regulate the spatial and temporal patterns of transgene expression tends to increase the success of the application of transgenic technology. Over the years, several promoters have been isolated from a wide variety of organisms and applied to genetically engineered plant systems (Potenza *et al*., 2004).

The promoter will mostly regulate the transgene expression, since the transcription process is the first gene regulation. However, expression of the transgene is not uniform in all plants generated under the same conditions as it is subjected to the endogenous regulatory mechanism of the plant. This variability of expression can be reduced by choosing an appropriate promoter to regulate the transgene, improving the efficiency of the technique (Butaye *et al*., 2005).

Current knowledge about the structure and functions of promoters in eukaryotic systems was recently reviewed by Porto *et al*. (2014). These researchers also describe the strategies used to isolate and analyze promoters and procedures available to estimate their expression.

Promoters can be classified as constitutive, inducible and organ or tissue-specific. A constitutive promoter directs the expression of a gene in all tissues of a plant during the various stages of development. A tissue-specific promoter directs expression of the gene only in certain tissues and may or may not be activated during all stages of development. An inducible promoter initiates gene expression in response to chemical, physical or biotic and abiotic stresses (Carneiro and Carneiro, 2011).

CONSTITUTIVE PROMOTERS

The promoters usually used in the production of genetically modified plants include the 35S promoter of the Cauliflower Mosaic Virus (CaMV 35S) and the promoter of the virus gene encoding Ubiquitin (Ubi-1) of maize (Hoshino, 2007). In particular, the CaMV 35S promoter is valuable because it provides high expression in all regions of the transformed plant and is generally available in the cassette vector used for transformation which facilitates the sub-cloning of the transgene of interest (Potenza *et al*, 2004).

Several other promoters that may be used for genetic transformation come from different organisms. In the case of constitutive promoters derived from the genome of viruses, promoters of several viruses may be used in addition to CAM 35 S: CAM 19 (Driesen *et al*., 1993), the Mirabilis Mosaic Virus (MMV) (Dey and Maiti, 1999) and the Strawberry Vein Banding Virus (SVBV) (Pattanaik *et al*., 2004). Among the constitutive promoters from bacteria the promoters of *nos* gene (Shaw *et al*., 1984) and *ocs* (Ellis *et al*., 1987) can be mentioned which respectively encode the nopaline synthase and octopine synthase both of *Agrobacterium tumefaciens*.

In the case of plants, the most widely used constitutive promoters are the promoter of the gene encoding ubiquitin of maize (Ubi-1) (Christensen and Quail, 1996) and the actin gene from rice (*Act1*) (McElroy *et al*., 1990).

However, the use of constitutive promoters causes unnecessary gene expression increasing the possibility of interference with other routes of plant development (De Paoli *et al*., 2007). Some negative characteristics of the use of constitutive promoters have been observed, highlighting mainly phenotypic changes in transformed plants (Matsuhara *et al*., 2000). In a study performed on *Solanum tuberosum* differences were found when a constitutive promoter (CaMV 35S) or a stress inducible rd29A promoter expression were used to drive *Atcbf* genes. In this case, the same level of freezing tolerance was observed both in plants containing the constitutive promoter and in plants containing the inducible promoter when exposed to cold for a few hours. However, in the plants containing the *Atcbf* gene regulated by the constitutive promoter, the leaves showed reduced size, retarded flowering and reduction and/or lack of tuber production (Pino *et al*., 2007).

Therefore, plant promoters that are activated specifically when and where needed are ideal for genetic engineering applications (Potenza *et al*., 2004).

INDUCED PROMOTERS

Promoters that are induced under certain stress conditions, both biotic and abiotic are interesting biotechnological tools for use in plant breeding programs. In general, the stress-inducible promoters contain a cis-acting sequence which is recognized by specific transcription factors that induce the synthesis of proteins only under conditions of stress (Jaglo *et al*., 2001). The

Table 1: Examples of promoters induced by abiotic stress

Correspnding gene	Inducer	Organism	References
HSP18.2	Thermal shock	*Arabidopsis thaliana*	Takahashi *et al.* (1992)
Rd29	Osmotic stress	*Arabidopsis thaliana*	Yamaguchi-Shinozaki and Shinozaki (1993)
adh	Dehydration and cold stress	*Arabidopsis thaliana*	Dolferus *et al.* (1994)
rbcS-3A	Light	*Pisum sativum*	Kuhlemeier *et al.* (1989)
Chn48	Ethylene	*Nicotiana tabacum*	Shinshi *et al.* (1995)
PvSR2	Heavy metals	*Phaseolus vulgaris*	Qi *et al.* (2007)
cgmt1	Heavy metals	*Casuarina glauca*	Laplaze *et al.* (2002)
HVADhn45	Drought stress	*Hordeum vulgare*	Xiao and Xue (2001)
PtDrl02	Methyl jasmonate	*Populus* sp.	Zheng *et al.* (2011)

Table 2: Promoters induced by biotic stress

Promoters	Inducer	Organism	References
CaPrx	Nematode infection	*Coffea arabica*	Severino *et al.* (2012)
R2329 and R2184	Blast fungus infection	*Oriza sativa*	Sasaki *et al.* (2007)
OsNAC6	Fungus infection	*Oriza sativa*	Nakashima *et al.* (2007)
PPP	Pathogens	*Arabidopsis* sp.	Peng *et al.* (2004)

Germin-Like (GLP) proteins with various functions in the development and protection of plants are also related to inducible promoters. One of them is the ThGLP promoter isolated from *Tamarix hispida* which was highly induced by drought, salt, low temperature and treatment with abscisic acid, its expression occurring in leaves and roots (Li *et al*., 2010). Table 1 lists some promoters induced by abiotic stress.

Biotic stress-induced promoters also deserve attention because they are just as important as the promoters induced by abiotic stress. Among the most studied are the promoters induced by pathogens that are quickly activated in response to stress and are effective in the plant defense process (McDowell and Woffenden, 2003).

A well-studied inducible stress promoter is Gst1 promoter from potato which activates gene transcription in response to infection by bacterial and fungal pathogens in transgenic apple (Malnoy *et al*., 2006). In transgenic citrus plants, the same promoter promoted gene expression in response to injury or to the pathogen *Xanthomonas axonopodis* ssp. *citri* (Barbosa-Mendes *et al*., 2009). Another promoter that has an important role in the plant defense system is the promoter belongs to class 10 PR (pathogenesis related). Coutos-Thevenot *et al*. (2001) related the combination of this pathogen-inducible promoter and a defense gene, the *Vst1* gene which may increase tolerance against fungi in grape vine.

In order to improve pear resistance against fire blight caused by *Erwinia amylovora*, a search for promoters driving high-level expression of transgenes specifically in response to this bacterial pathogen has been undertaken. Malnoy *et al*. (2003) examined the ability of hsr203J, str246C and sgd24 promoters of tobacco (*Nicotiana tabacum* L.) to drive expression of the *uidA* reporter gene in transgenic pear. It was demonstrated that two of them (*str246C* and *sgd24*) were functional in pear, a woody species botanically distant from tobacco and activated by wounding and elicitors. They could therefore be used to drive the expression of transgenes to promote bacterial disease resistance (Table 2).

SPECIFIC PROMOTERS

The use of organ or tissue specific promoters that induce and specifically control the expression of transgenes in organ and/or tissue may be advantageous to avoid a waste of energy and nutrients from the transgenic plant when the protein of interest is not necessary for the whole plant. Furthermore, the use of these promoters is convenient in both the commercial and scientific contexts and provides increased biosecurity, among other advantages the isolation and characterization of appropriate promoters for plant genetic engineering is therefore highly desirable (Daniell, 2002; Potenza *et al*., 2004; Carneiro and Carneiro, 2011).

There are several promoters of viral, microbial and plant origin able to direct organ-specific expression in plants; however it is desirable that these promoters originate from the same plant species orphylogenetically related species because the regulatory systems are unique and cannot act in the expected manner in distant heterologous species (Tyagi, 2001).

ROOT-SPECIFIC PROMOTERS

Root-specific promoters are of particular interest, since they promise a wide variety of applications. Recombinant proteins can be expressed for almost anything that is related to the root-soil interface using genetic engineering for bioremediation of soil contaminants, protection against drought, increased salt tolerance, capture of macro and micronutrients and

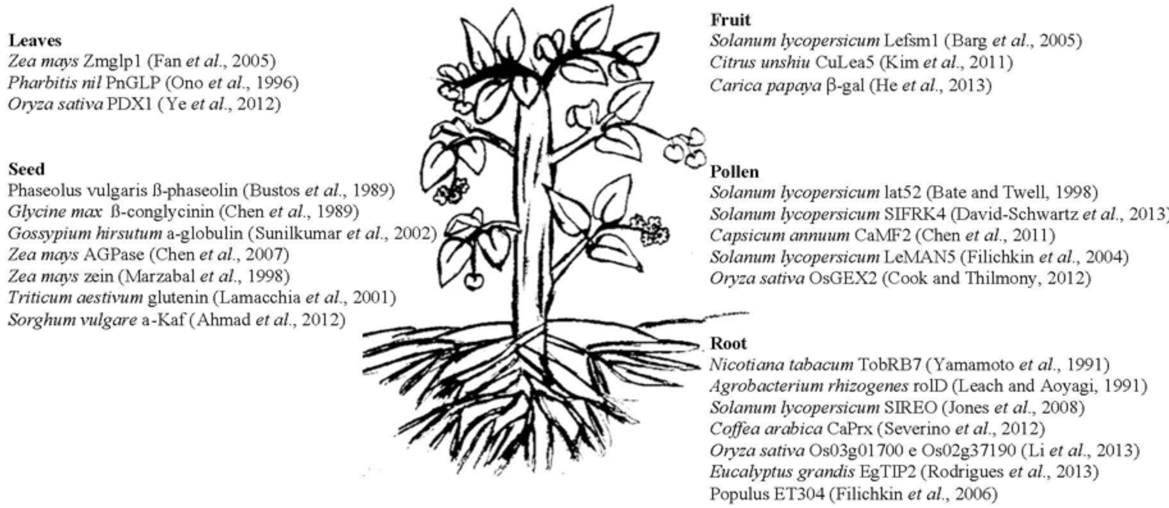

Fig. 1: Examples of organ/tissue specific promoters in some plant species

increased resistance to root pathogens (Potenza *et al.*, 2004). In addition, expression of the transgene in the root can be specifically suitable for the use of marginal soils (Twyman, 2003).

The root is the first organ of the plant that feels ionic, osmotic and other stresses resulting from drought, soil salinity, accumulation of heavy metals, nutrient deficiency and the presence of microorganisms in the rhizosphere (Jones *et al.*, 2008). Furthermore, the root system captures water and nutrients, important for the development and crop yield; therefore, there is a growing concern about studies on root-specific and root inducible promoters since the overexpression of proteins located in roots is able to improve the growth or stress tolerance of plants (Ghanem *et al.*, 2011).

Some root-specific promoters have been identified and isolated (Fig. 1) such as the root-specific promoter of tobacco gene *TobRB7* whose expression was found in the root meristem and immature central cylinder regions (Yamamoto *et al.*, 1991). The promoter of the *rolD* gene from *Agrobacterium rhizogenes* showed high expression in roots and little expression in leaves of transformed Arabidopsis (Leach and Aoyagi, 1991) and it was used for the specific root expression of the glutamine synthetase gene (Fei *et al.*, 2003). Other promoters of this type are: the promoter of the *SlREO* gene with high expression in tomato roots (Jones *et al.*, 2008), the promoter of the *CaPrx* gene of *Coffea arabica* specifically expressed in roots and in response to biotic stress and infection by nematodes in early stages (Severino *et al.*, 2012) and the Os03g01700 and Os02g37190 promoters of rice genes characterized by Li *et al.* (2013) that are highly active and possess the ability to induce high levels of expression in root tissues.

Few root-specific promoters were studied in forest species but the following stand out: the *PmPR10-1.14* gene promoter of *Pinus monticola* that directed the gene expression in root tissues of transgenic tobacco (Liu and Ekramoddoullah, 2003), the promoter of the gene *EgTIP2* (root-specific tonoplast intrinsic aquaporin) of *Eucalyptus grandis* that exhibits expression in vascular tissues of the whole plant and root tips of transgenic tobacco (Rodrigues *et al.*, 2013) and the promoter of the *ET304* gene of poplar conferring strong expression in roots of transgenic poplar and Arabidopsis (Filichkin *et al.*, 2006).

SEED AND FRUIT SPECIFIC PROMOTERS

In grain producing plants, genes encoding reserve proteins are highly expressed during seed development and promoters of these genes have been used in genetic engineering experiments. The seed-specific expression of transgenes has also been used to increase the production of pharmaceutical or industrial compounds and enhance the functional and nutritional quality of grain (Ye *et al.*, 2000).

The promoters used in most dicots are the promoters of the β-phaseolin gene from beans (Bustos *et al.*, 1989) and of the β-conglycinin gene of soybean (Chen *et al.*, 1989). The promoters of α or α 'subunits of β-conglycinin protein reserves were also used for seed-specific gene silencingand can cause a small increase in amino acid content in soybean (Kim *et al.*, 2014).

Other seed-specific promoters were characterized, among them the α-globulin promoter in cotton (Sunilkumar *et al.*, 2002), gamma-zein promoters in maize

(Marzabal *et al.*, 1998), the promoter of glutenin genes in wheat (Lamacchia *et al.*, 2001) and the promoter of the α-*Kaf* gene in sorghum (Ahmad *et al.*, 2012).

Fruit-specific promoters were also cloned and tested in several plants (Fig. 1): in tomato, *Lefsm1* gene promoter is specific to the early stages of fruit development (Barg *et al.*, 2005) in the case of *Citrus unshiu*, CuLea5 promoter confers preferential expression in the fruit and its expression is enhanced by plant hormones such as abscisic acid and naphthalene acetic acid and abiotic stresses such as cold and drought (Kim *et al.*, 2011). In addition, a fruit pulp specific promoter was identified by He *et al.* (2013), it is the promoter of the *β-gal* gene that is related to the softening of papaya pulp. This promoter was used in a silencing construction in order to reduce the softening of the fruit of this species, thereby lengthening its storage life.

Figueroa *et al.* (2009) observed that the promoter of the *FaExp2* strawberry gene has a high level of expression in the fruit during ripening. Later on, in order to achieve the specific expression of transgenes in strawberry fruit, Schaart *et al.* (2011) isolated 5'upstream sequences of the *FaExp2* gene. Two different lengths of promoter fragments (0.7 and 1.6 kb) were isolated and characterized and a standard specific expression in the fruit achenes was observed in transformed plants. Researchers are investigating the suitability of the 1.6pFaExp2 promoter to direct the expression of an antifungal gene with the aim of increasing resistance to fruit rot caused by *Botrytis cinerea.*

POLLEN-SPECIFIC PROMOTERS

Some pollen-specific promoters have been identified and characterized (Fig. 1) including the promoter of tomato *lat52* gene encoding a cysteine-rich protein preferentially transcribed in vegetative cells during maturation of the pollen (Bate and Twell, 1998). Another isolated promoter is the promoter of tomato *SlFRK4* gene that is stamen-specific and responsible for the metabolism of fructokinase 4. This promoter is gradually activated in pollen grains during the final stages of anther development and upon pollen germination of transformed plants of *Arabidopsis thaliana* (David-Schwartz *et al.*, 2013).

It is important to use specific promoters in pollen gene silencing in order to prevent the flow of genes from transgenic plants: this was the case of the promoter of the gene *CaMF2* where inhibition of the promoter by Virus Induced Gene Silencing (VIGS) resulted in low germination of pollen grains of *Capsicum annuum* (Chen *et al.*, 2011). Another example is the promoter of *LeMAN5* tomato gene with an endo-β-mannanase function that is expressed in anthers and pollen during development and can be used to control pollen fertility and to increase the production of hybrid seeds (Filichkin *et al.*, 2004).

SPECIFIC PROMOTERSOF LEAVES AND VASCULAR TISSUES

Leaf specific promoters are used most often to direct the expression of genes only in leaves for disease control and when the expression of certain genes is not desired in other organs, especially in fruits (Fig. 1). Some promoters direct the expression of genes in young leaves: the promoter of the gene *Zmglp1* ("Germin-like protein") in maize caused abundant expression in new leaves and more abundant in mature leaves (Fan *et al.*, 2005) and the promoter of the gene *PnGLP* did the same in new leaves and cotyledons of *Pharbitis nil* (Ono *et al.*, 1996).

With regard to specific vascular tissue promoters, Lauvergeat *et al.* (2002) studied the promoter of *Eucalyptus gunnii* gene *EgCAD2* whose use in the composition of expression cassettes allows direct transgene expression in vascular tissues of perennial (vine and poplar) and herbaceous (tobacco) plants. Another successful example is the promoter EgCCR of *Eucalyptus gunnii* which directed transgene expression in vascular tissue of the vine (Gago *et al.*, 2011). Both EgCAD2 and EgCCR promoters may contribute to an important application of genetic engineering which would drive the expression of defense genes to vascular tissue in order to increase vascular resistance to pathogens. This new trait is extremely valuable for plants of economic interest throughout the world (Lauvergeat *et al.*, 2002; Gago *et al.*, 2011).

In citrus, Dutt *et al.* (2012) studied the use of phloem-specific rolC promoters from *Agrobacterium rhizogenes* as antibacterial constructs created to combat huanglongbing (HLB or citrus greening disease), associated with a phloem-limited Gram-negative bacteria where the Rice Tungro Bacilliform Virus promoter (RTBV) showed high levels of expression of the *gus* gene in citrus.

Another recently characterized promoter is the promoter of the *Athspr* gene of heat shock protein from *Arabidopsis thaliana* which showed expression in vascular tissues in all organs of the transformed plants indicating that this promoter has multiple roles in vascular development and can be used to obtain plants resistant to various other stresses (Zhang *et al.*, 2014).

CONCLUSION

Due to the increase in research related to genetic transformation biosafety, concerns are arising and researchers have carried out studies of gene expression in specific tissues and organ and have made efforts to isolate promoters adding value to transgenes. The applications of tissue/organ promoters are numerous and the use of induced promoters to minimize the negative effects of unfavorable environmental conditions such as drought, saline soils, low and high temperatures can be emphasized.

Induced and organ/tissue specific promoters will activate the expression of genes only in specific situations which will reduce the energy expenditure of the plant as it will be activated only when really needed. In this way, productivity may be maintained, even under unfavorable environmental conditions.

On the other hand, the pollen-specific promoters can be used preferentially to control gene flow from transgenic plants, minimizing the risk of crossing between non transgenic and genetically modified plants and increase environmental security.

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