Enhancement of Somatic Intrachromosomal Homologous Recombination in Arabidopsis by the HO Endonuclease

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The HO endonuclease promotes gene conversion between mating-type alleles in yeast by a DNA double-strand break at the site of conversion (the MATYR site). As a first step toward understanding the molecular basis of homologous recombination in higher plants, we demonstrate that expression of HO in Arabidopsis enhances intrachromosomal recombination between inverted repeats of two defective β-glucuronidase (gus) genes (GUS-test construct). One of these genes has the Y/Z site. The two genes share 2.5 kb of DNA sequence homology around the HO cut site. Somatic recombination between the two repeats was determined by using a histochemical assay of GUS activity. The frequency of GUS+ sectors in leaves of F1 plants from a cross between parents homozygous for the GUS-test construct and HO, respectively, was 10-fold higher than in F1 plants from a cross between the same plant containing the GUS-test construct and a wild-type parent. Polymerase chain reaction analysis showed restoration of the 5' end of the GUS gene in recombinant sectors.

The induction of intrachromosomal gene conversion in Arabidopsis by HO reveals the general utility of site-specific DNA endonucleases in producing targeted homologous recombination in plant genomes.

INTRODUCTION

A fundamental difference between plants and animals is that in plants, there is no germ line set aside in early development. Somatic recombination events are potentially heritable (Das et al., 1990) and are therefore important in evolution. Because experimental gene replacement by somatic recombination has not been feasible in higher plants, it is important to investigate the molecular mechanisms of somatic recombination in Arabidopsis. DNA double-strand breaks (DSBs) are natural initiators of homologous recombination in Escherichia coli (Kobayashi, 1992), phage λ (Myers and Stahl, 1994), and fungi (Strathern et al., 1982; Sun et al., 1989; Cao et al., 1990; de Massy et al., 1995; Liu et al., 1995). Genetic analysis of transposon excision implicates DSBs as initiators of homologous recombination in maize (Athma and Peterson, 1991), Caenorhabditis elegans (Plasterk and Groenen, 1992), and Drosophila (Gloor et al., 1991).

I-SceI, a yeast site-specific endonuclease, can enhance homologous recombination between transiently introduced extrachromosomal DNA in tobacco (Puchta et al., 1993). Similarly, I-SceI can stimulate both extrachromosomal and intrachromosomal recombination in mammalian cells (Rouet et al., 1994; Choulika et al., 1995). In yeast, the site-specific endonuclease HO, which recognizes and cleaves the 24-bp MAT-YR target site (Kostriken et al., 1983; Nickoloff et al., 1986), stimulates intrachromosomal homologous recombination between repeated DNA elements (Strathern et al., 1982; Nickoloff et al., 1986; Ray et al., 1988; Rudin and Haber, 1988). The broken chromosome is repaired by homologous recombination with an uncleaved donor allele in a gene conversion process. In plants, spontaneous somatic recombination, selected and unselected, between artificial repeat elements has been documented (Assaad and Signer, 1992; Tovar and Lichtenstein, 1992; Swoboda et al., 1994; Puchta et al., 1995).

The molecular nature of the recombination-initiating lesion in plants is not known. This prompted us to investigate whether a defined chromosomal DSB can stimulate somatic homologous recombination in a plant. We now report that in Arabidopsis, the HO endonuclease induces intrachromosomal homologous recombination between two unselected elements forming an inverted repeat (Figure 1A). An inverted repeat was chosen because recombination between direct repeats can...
RESULTS

Experimental Strategy

We constructed an inverted repeat of two mutant heteroallelic β-glucuronidase (gus) genes. It was designated gus3Δ-P35S-pUC19-P35S-5′gus::y/z, and we refer to it as the GUS test construct (Figure 1A). The 5′gus::y/z allele was fused to the cauliflower mosaic virus (CaMV) 35S promoter (P35S) region extending from positions 5850 to 7440 bp in the CaMV sequence (Guilley et al., 1982). In this mutated gus allele, a 406-bp GUS region extending from positions -17 to +389 bp relative to the GUS gene transcription start site (Jefferson et al., 1987) has been replaced by a 124-bp fragment of the yeast MAT-Y/z junction sequence containing the 24-bp target site of the yeast HO endonuclease (Nickoloff et al., 1986). The gus3Δ allele contains the typical 5′ end of the GUS gene fused to the P35S promoter region extending from positions 6107 to 7440 bp in the CaMV sequence (Guilley et al., 1982) but has a deletion that removes 3′ sequences distal to position +1640 in the GUS gene (Jefferson et al., 1987). The overall sequence homology between the two copies extends over 1333 bp on one side of the HO target site along the CaMV sequences and over 1251 bp on the other side along the gus sequences. Both mutant gus alleles, being large deletions, are nonrevertible. This inverted repeat construct was introduced into Arabidopsis by Agrobacterium-mediated transformation (see Methods). A line homozygous for a single chromosomal insert of the construct was selected on the basis of DNA gel blot analysis, shown in

accompanying the gap repair will lead to an inversion of the intervening sequence.

(B) A nonreciprocal break-join event may also restore the GUS gene sequences by attaching the left half of gus-y/z to the right half of gus-3Δ sequences. This latter process should produce chromosome fragmentation that must be healed by an illegitimate recombination event.
HO-Induced Somatic Recombination in Arabidopsis

To determine the rate of spontaneous somatic recombination in the unique copy of the inverted gus locus, we crossed lines descending from ARP4 to wild-type Arabidopsis ecotype RLD plants. Leaves of F1 plants were stained to reveal blue Gus+ sectors. Stained leaves of these F1 plants contained rare, minute (typically spanning 10 to 50 cells) blue sectors (mean = 0.29 ± 0.078 sectors per leaf, with 66 leaves on 22 plants examined). A representative leaf is shown in Figure 3A. The frequency distribution of these sectors approximated (by the goodness of fit test χ² = 5.53, P > 0.05) a Poisson distribution of the same mean, as shown in Figure 4A. Thus, spontaneous Gus+ sectors were rare and randomly distributed among leaf cells. The mean frequency of spontaneous recombination was of the same order of magnitude as that seen by others (Swoboda et al., 1994; Puchta et al., 1995). Because these spontaneous sectors were small compared with the total leaf size, the recombination events must have occurred

Figure 2A, and a Mendelian segregation pattern. This line is designated ARP4.

In the event of a DSB at 5′Δgus::y/z, gus3′Δ could serve as the donor sequence in a gene conversion process. This DSB was expected to generate a wild-type GUS allele (Figure 1A), whose expression in plant tissues could be detected by a standard staining assay with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (Jefferson, 1987). Polymerase chain reaction (PCR) analysis of DNA from stained and unstained sectors would provide additional molecular evidence of the repair process. Expression of HO was under the control of either the constitutive CaMV 35S promoter (P35S-HO) or a Drosophila 70-kD heat shock promoter (Phsp70-HO) (Spena et al., 1985). The effect of HO on recombination at the Gus inverted repeat was examined under two separate conditions. First, plants containing the GUS+ test construct were crossed with plants containing P35S-HO, and the F1 leaves were examined for recombination in the Gus+ test construct by staining for Gus+. Second, plants carrying the GUS+ test construct were crossed with plants containing Phsp70-HO; F1 plants and calli prepared from their leaves were heat shocked, and DNA from the calli was analyzed by PCR to detect recombination.

Spontaneous Recombination at the Inverted gus Repeat

To determine the rate of spontaneous somatic recombination in the unique copy of the inverted gus locus, we crossed lines descending from ARP4 to wild-type Arabidopsis ecotype RLD plants. Leaves of F1 plants were stained to reveal blue Gus+ sectors. Stained leaves of these F1 plants contained rare, minute (typically spanning 10 to 50 cells) blue sectors (mean = 0.29 ± 0.078 sectors per leaf, with 66 leaves on 22 plants examined). A representative leaf is shown in Figure 3A. The frequency distribution of these sectors approximated (by the goodness of fit test χ² = 5.53, P > 0.05) a Poisson distribution of the same mean, as shown in Figure 4A. Thus, spontaneous Gus+ sectors were rare and randomly distributed among leaf cells. The mean frequency of spontaneous recombination was of the same order of magnitude as that seen by others (Swoboda et al., 1994; Puchta et al., 1995). Because these spontaneous sectors were small compared with the total leaf size, the recombination events must have occurred

with primers 3 and 4 (see Figure 1 and Methods) from size-selected DNA fragments of Gus+ calli are shown in lanes 2 and 3. Template fragments were sized from 3.6 to 3.4 kb (lane 2) and from 3.2 to 3.4 kb (lane 3). The faint band in lane 3 is most likely due to a slight contamination during size selection with the 3.5-kb Gus recombinant band (see text). Lane 4 contains the PCR product obtained from template DNA fragments, sized from 3.6 to 3.2 kb, that were derived from Gus+ calli. Lanes 1 and 5 contain the DNA length markers. DNA marker lengths (in kilobases) are indicated at left.
Figure 3. HO-Induced Homologous Recombination in Transgenic Plants.

The first three leaves of F1 progeny from crosses (see text) were assayed for Gus+ activity. Dark sectors on leaves represent blue Gus+ spots.
(A) A leaf of an ARP4 x RLD F1 control cross. This leaf showed two minute Gus+ sectors.
(B) and (C) Leaves from ARP4 x AL66-1 F1 crosses. Note many small recombinant sectors and a few larger ones.
(D) Recombinant sectors in a heat-shocked callus derived from an ARP4 x HS104-1 F1 leaf.

in postembryonic seedlings during leaf formation. Therefore, each sector should represent an independent recombination event.

Intrachromosomal Recombination Induced by an Integrated P35S-HO Gene

To induce homologous recombination by HO in the plants, we crossed kanamycin-resistant lines descending from the homozygous transgenic line ARP4 with kanamycin-resistant descendants of the line AL66-1 that were homozygous for the transgene P35S-HO.

Stained leaves of ARP4 x AL66-1 F1 plants contained on average 10 times more blue sectors (3.06 ± 0.435 sectors per leaf, with 64 leaves on 22 plants examined) than did leaves of control ARP4 x RLD F1 plants (Figure 4). These data are significantly different from the control, as tested by the median test ($\chi^2 = 16.8, P << 0.01$). The blue recombinant sectors in ARP4 x AL66-1 F1 leaves were usually larger (typically spanning >100 cells) than those observed in control crosses (Figures 3B and 3C). Furthermore, the frequency distribution of Gus+ sectors in leaves of ARP4 x AL66-1 crosses did not fit a Poisson distribution of the same mean (by the goodness of fit test, $\chi^2 = 92.0, P << 0.01$; Figure 4A). Thus, Gus+ sectors were not rare and were not randomly distributed among the leaf cells. In particular, there were significantly more leaves containing more than eight Gus+ sectors than were expected from a Poisson distribution. Likewise, there were more leaves with fewer than two sectors than were expected for randomness, but these rare sectors were much larger than those in spontaneous controls (Figure 3C). Moreover, the plants were heterogeneous for induction of recombination (Figure 4B). In general, if one leaf had more than five sectors, then another leaf from the same plant tended to have more than five Gus+ sectors as well. Most Gus+ sectors were restricted to the vasculature or, more rarely, to the epithelial cells, including the stomata. A few large sectors were associated with mesophyll...
cells. The vasculature and the epithelial cells are derived from different clonal cell layers of the meristem. Thus, we suggest that the sectors were most likely clonal, although this conclusion should await more extensive mosaic analysis.

DNA preparations from leaves of plants that contained GUS+ sectors on other leaves were analyzed by DNA gel blot hybridization for the presence of the recombinant GUS gene (Figure 2A). Recombinant GUS bands could not be detected even after long exposures, presumably because the recombinant DNA was a minor fraction of the total DNA in these leaves.

**PCR Analysis of GUS+ Sectors in Leaves**

To confirm the presence of a recombinant GUS gene in blue sectors, we excised adjacent blue and non-blue leaf sectors, and their DNA was amplified by PCR using primers 1 and 2 (Figure 1A), which are specific for the 5' and 3' deleted regions of GUS, respectively. Such primers should not amplify any signal from the parental gus mutant genes but should amplify a restored GUS fragment (Puchta et al., 1995). A 1442-bp PCR signal diagnostic of a recombinant GUS gene was obtained from all blue and most non-blue sectors of ARP4 × AL66-1 plants (Figure 2B). For these experiments, DNA from ~10 times more non-blue sector area than that of its neighboring blue sector was used for PCR. Even so, the PCR signal from the non-blue sector, when present, was consistently less intense than that from the neighboring blue sector. By contrast, the corresponding PCR signal was not detected in DNA isolated from AR4 (Figure 2B) or AL66-1 parents (data not shown), suggesting that the PCR signal from non-blue sectors represented either the presence in non-blue sectors of recombinant cells not detectable by the Gus staining assay or contamination with recombinant GUS DNA from adjacent blue-sector cells. We favor the latter possibility because GUS+ cells are usually detectable at a single-cell resolution. In conclusion, the blue sectors represent recombinant cells, but we cannot rigorously eliminate the possibility of some recombinant cells in adjacent non-blue sectors.

**Intrachromosomal Homologous Recombination Induced by HO Expression from a Heat Shock Promoter**

To increase the production of recombinant GUS genes, we generated a transgenic plant line (HS104-1) expressing P_E70-HO. Steady state HO mRNA expression in this line, as measured by RNA gel blot analysis, was strongly inducible by heat shock at 37°C but was undetectable at the typical growth temperature (21°C) (Figure 2C). The steady state HO mRNA level was detectable immediately after 15 min at 37°C, rose to a maximum in 60 min, and remained at that level (normalized over tubulin mRNA) for at least 2 hr.

To test whether high levels of heat shock–induced HO expressed in HS104-1 could induce recombination at the GUS–cassette, we crossed HS104-1 to ARP4. F1 seedlings were heat shocked at 37°C for 2 hr, transferred to the typical growth temperature, and further incubated to allow leaf expansion. Leaves of F1 plants with or without heat shock treatment were stained for GUS activity. None of the six non-heat-shocked F1 plants revealed any GUS+ sectors in their leaves. One GUS+ sector was seen in six parental ARP4 plants that were treated similarly by heat shock. In contrast, of 20 leaves of 10 heat-shocked F1 plants derived from an HS104-1 × ARP4 cross, 14 leaves had many blue sectors. Thus, heat shock was correlated with an increased frequency of blue sectors in the F1 progeny. Repeated cycles of 2 hr of heat shock over several days did not increase the GUS+ sector frequency any more than did one 2-hr heat treatment. It is possible that HO-induced recombination is restricted to a small window of time during plant development. Nevertheless, in attempts to generate sufficient recombinant tissues for physical analysis, we produced and periodically heat shocked (at 37°C for 2 hr on every second day for 7 to 21 days) leaves and the calli generated from progeny of the ARP4 × HS104-1 crosses (Figure 3D). Despite our best efforts, we were unable to detect recombinant bands by gel blot hybridization with DNA prepared from these blue calli because the small recombinant sectors yielded insufficient quantities of DNA. Therefore, we performed PCR analysis on size-selected DNA to confirm the recombinants.

PCR amplification of total DNA isolated from non-blue or blue sectors in such calli using primers 3 and 4 bracketing the Y/Z site (Figure 1A) yielded two bands of 300 and 550 bp, respectively. The 300-bp band was expected to signal the presence of the parental Δ5'gus;Δ3' unit; the 550-bp band may have been due to either the parental gusA3' unit or the recombinant GUS gene. Although the 550-bp band was expected from both blue and non-blue sectors, the 300-bp band was expected from only the non-blue sectors. Thus, the 300-bp signal from blue sector DNA could represent a few nonrecombinant cells that must invariably be present in these preparations because there was no selection against non-recombinant cells.

The identity of these bands was further confirmed by digestion with restriction endonucleases (BamHI and HindIII) predicted to have specific sites within these fragments. As predicted for the parental gusA3' unit and the legitimate GUS recombinants, the 550-bp band was cleaved by BamHI but not by HindIII. Conversely, the 300-bp band was cleaved by HindIII but not by BamHI. A potential alternative source of an ~300-bp signal is cells in which an illegitimate joining of the broken junction has occurred such that the internal Y/Z fragment is partially deleted. In these cases, one or both of the HindIII sites would be lost. If both HindIII sites were lost, the PCR signal should be at most 176 bp, not 300 bp. Because such signals were not detected, it is unlikely that the blue recombinant sectors had much illegitimate activity around the Y/Z junction sequence.
To distinguish between the presence of recombinant GUS DNA and the parental gusA3' unit, we sampled the same genomic DNA samples with EcoRI and HindIII and separated them on an 0.8% agarose gel. Double digestion releases the parental Δ5'gus::Y/z repeat unit as two fragments of 1.3 and 1.8 kb and the parental gusA3' repeat unit as a 3.0-kb fragment. However, a recombinant GUS unit with the promoter fragment should be released as a single 3.5-kb band. Regions of the agarose gel that corresponded to DNA fragments ranging from 3.2 to 3.3 kb and from 3.4 to 3.6 kb were cut out separately. DNA extracted from these gel pieces was separately subjected to PCR analysis with primers 3 and 4.

PCR amplification of size-selected DNA from blue calli revealed a 550-bp band (Figure 2D, lanes 2 and 3). This is expected for a restored GUS gene but not for a parental gusA3' unit because the two HindIII sites flanking the MAT-Y/z insertion point in the GUS+ test construct should be replaced by 5' GUS sequences having no HindIII sites (Figure 1). If HO cutting at Δ5'gus::Y/z were to provoke deletion of the two HindIII sites followed by illegitimate ligation, an amplified signal of <300 bp would be expected. This was never obtained. Thus, illegitimate recombination did not lead to a loss of the two HindIII sites. Furthermore, the 550-bp PCR band was never obtained with EcoRI-HindIII-digested, 3.2- to 3.6-kb size-fractonated DNA from non-blue calli (Figure 2D, lane 4). In other words, the parental gusA3' unit that might have generated a 550-bp signal could not have been present in the 3.2- to 3.6-kb size class. This shows that the 550-bp signal obtained from size-fractonated blue-sector DNA represented the recombinant GUS gene.

The 550-bp PCR signal was consistently stronger when PCR was performed with larger DNA species from blue calli ranging from 3.4 to 3.6 kb (Figure 2D, lane 2) than with smaller DNA species ranging from 3.2 to 3.3 kb (Figure 2D, lane 3). The 3.5-kb recombinant GUS DNA fragment should be present in the 3.4- to 3.6-kb fraction but not in the 3.2- to 3.3 kb fraction. Therefore, the weak signal in the latter case is most likely due to slight DNA degradation from the 3.5-kb recombinant band, not from a contamination with the 3.0-kb parental gusA3' band. Furthermore, the genomic DNA template was probably completely digested; partial digestion with EcoRI and HindIII would have created a 3.2-kb EcoRI fragment comprising the Psse-5'S5'gus::Y/z region (Figure 1A), which when subjected to PCR would have produced a 300-bp amplified DNA fragment that in fact was never detected (Figure 2D).

We conclude that intrachromosomal recombination induced by the HO endonuclease restores the GUS gene in blue sectors. In addition, we conclude that most recombination events in the blue sectors are true gap repair because we found no evidence for illegitimate deletion of the Y/Z junction region. The possibility of illegitimate events in non-blue sectors, however, is not entirely eliminated by these experiments. Because DNA gel blot analysis could not be performed, we were unable to address the question of associated crossover with these gene conversion events. A crossover would have inverted the pUC19 moiety with respect to the two gus repeats. Attempts to detect associated crossover by restriction analyses on long-range PCR products with primers 1 and 3 were unsuccessful.

DISCUSSION

We have demonstrated that expression of the site-specific HO endonuclease in Arabidopsis enhances somatic homologous recombination between inverted repeats in the chromosome. Several recent studies have provided an analysis of spontaneous recombination at repeated genes in plants (Gal et al., 1991; Assaad and Signer, 1992; Tovar and Lichtenstein, 1992; Swoboda et al., 1994; Puchta et al., 1995). It is not clear at present whether spontaneous mitotic or meiotic recombination in plants is initiated by a DNA DSB, as it is during yeast meiosis (Wu and Lichten, 1994; de Massy et al., 1995; Liu et al., 1995; Schwacha and Kleckner, 1995). Past studies with yeast have provided the ground rules for spontaneous homologous recombination at repeated DNA (Jackson and Fink, 1981, 1985; Klein, 1984; Petes and Hill, 1988), which were later found to be satisfied in HO-induced homologous recombination at chromosomal repeats (Ray et al., 1988). Our experiments represent the beginning of such studies with Arabidopsis as a model for the molecular basis of homologous recombination in plants.

The observed HO-induced homologous recombination that restored the GUS gene may have occurred by the DSB repair (DSBR) mechanism (Szostak et al., 1983) or by the synthesis-dependent single-strand annealing (SDSA) mechanism (Lin et al., 1990; Fishman-Lobell et al., 1992; Nassif et al., 1994). These mechanisms are diagrammed in Figure 5. In principle, either model is sufficient to explain our observations. Both mechanisms could also account for the results in which recombination was induced between an I-SceI cleavable chromosomal recipient and a donor borne on a transiently introduced T-DNA (Puchta et al., 1996). In that case, however, it was not clear whether the donor T-DNA was single or double stranded. The exact configuration of the donor and the recipient DNA may critically influence the mechanism of recombination. For example, a nonreciprocal (one-ended) recombination is common in HO-induced plasmid recombination but is virtually absent during intrachromosomal recombination between directly repeated DNA in yeast (Ray et al., 1988; Haber, 1995).

Nonreciprocal recombination involving sister chromatids may produce chromosomal deletions or aneuploidy, with the consequent death of the recombinant cell. Thus, our observed frequency of GUS+ sectors could be an underestimation of the true frequency of HO-induced events. Such illegitimate recombination has been observed at one of the double-stranded ends by Puchta et al. (1996). As discussed in the Results section, we did not detect any evidence of illegitimate repair of the Y/Z junction in blue-sector cells. We cannot, however, eliminate the possibility that such events did not occur within non-blue areas of the leaves. This reservation does not detract from our
Figure 5. Mechanisms of DSB-Induced Recombination.

Cleavage of the chromosome at Y/Z, followed by exonuclease digestion, produces DNA single strands with 3' overhangs. In the double-strand break repair (DSBR) model (Szostak et al., 1983), both single-stranded ends invade the intact homologous duplex, gap repair occurs, and the intertwined DNA strands resolve to generate the converted GUS gene. In the synthesis-dependent single-strand annealing (SDSA) model (Nassif et al., 1994), which is an extension of previous proposals by Lin et al. (1990) and Fishman-Lobell et al. (1992), one of the two single-stranded ends (here arbitrarily chosen as the left end) invades the intact duplex, primes repair synthesis, dissociates, and finally anneals to the complementary right-hand single strand. Single-stranded gaps are filled in by further synthesis, leading to gene conversion.
gene sequences (pAR151). The 124-bp YZ site on a HindIII fragment (Kosterkine et al., 1983; Ray et al., 1988) was cloned into the unique HindIII site of pAR151 between the cauliflower mosaic virus (CaMV) 35S promoter (P35S) (Guitely et al., 1982) and the 5'-gus gene to give pAR162. To delete the 3' end of GUS, plasmid p35S-GUS-tnos was digested with HaelI, blunt-ended with T4 DNA polymerase, ligated to HindIII linkers, and digested with HindIII to generate the gus3' fragment, which was ligated to the HindIII site of pUC19 to give pAR164. The pAR164 HindIII fragment containing P35S-gus3' was inserted by ligation at a unique HindIII site between the left and right border sequences of the pGA-3Sh binary vector (Perez et al., 1989) to give pAR168. Finally, a partial EcoRI digest of pAR162 was cloned into a complete EcoRI digest of pAR168, yielding pAR173 containing the inserted repeat cassette gus3'P35S-PUC19-P35S-gus:yz (Figure 1).

pAL66 Construction

The HindIII-EcoRI restriction fragment of pH0-o-t (Russell et al., 1986) containing the HO coding region was cloned into the corresponding sites of pUC20B (March et al., 1984) to create pAL40. pAL40 was partially digested with AhaI, and linear DNA fragments were gel purified and cut with Smal. The resulting DNA fragments were again gel purified and ligated, and the pAL41 plasmid was obtained after screening for loss of the HO 5' untranslated sequences upstream of the AhaI site nearest to the ATG start codon. This cloning step removed all spurious 5' upstream ATG codons. The HO-containing EcoRI fragment from pAL41 was cloned into the EcoRI site of pMC85 (Stratagene, La Jolla, CA), thus creating pAL42, whose HO start codon is nearest the BamHI site in pBlueScript KS+ (confirmed by DNA sequencing). pAL42 was partially cut with BamHI and completely cut with Kpnl, and the ~2-kb HO fragment was ligated into the polylinker of pKYX75 (Schardl et al., 1987) at the BamHI-Kpnl sites to create pAL43. pAL43 was cut with XbaI, and the HO-containing fragment was cloned into the pKYX71 dephosphorylated XbaI site (Schardl et al., 1987; Lloyd et al., 1992) to create pAL66. pAL66 contains the HO coding sequence under the control of the CaMV 35S promoter in a binary T-DNA plant transformation vector.

pMC104 Construction

A 0.5-kb EcoRI-BamHI fragment from pEV-hs (Steller and Pirrotta, 1984) containing the Drosophila melanogaster 70-kD heat shock promoter (P70kD) (Pelham, 1982) was cloned at EcoRI-BamHI sites of pSK1, creating pMC65. The 0.22-kb HindIII-Kpnl fragment from pGRCAT (Schena et al., 1992), which includes the CaMV 3' polyadenylation signal (CaMV3pter), was cloned at the HindIII-Kpnl sites of pMC65 to generate pMC66. A 2.5-kb HindIII fragment from pH0-D6 (Russell et al., 1986) containing the HO coding region was cloned into the HindIII site of pMC66 to give pMC91. Finally, the P35S-HO-CaMV3pter region was isolated as a 3.3-kb Notl-Kpnl fragment, blunt-ended by T4 DNA polymerase, and ligated to the binary vector pMC88 (derived from pGA35Sh by the removal of the neomycin phosphotransferase NPTII coding and regulatory regions by HindIII and SacI partial digestion) in the unique Scal site to give pMC104.

Plant Transformation

Transgenic plants were constructed by the root transformation method (Marton and Browse, 1991). Arabidopsis thaliana ecotype Bensheim was infected with strain AGL1 of Agrobacterium tumefaciens (Lazo et al., 1991) carrying pAR173, and transgenic plants were selected on kanamycin. Six of 15 kanamycin-resistant lines were selected by polymerase chain reaction (PCR) analysis of DNA for the presence of CaMV P35S and GUS sequences. One line that had a single T-DNA insert (established by DNA gel blot analysis; Figure 2A) was selected, and progeny from this line were tested for the homozygosity of the T-DNA insert by segregation. One homozygous line, AP4, was used in experiments described here. Arabidopsis ecotype RLD was infected with Agrobacterium strain GV3101 containing pAL66 and the helper plasmid pMP90 (Wing et al., 1989), and transformants were selected on kanamycin. More than 10 independent transformants were confirmed to have the correct P35S-HO construct by PCR analysis (data not shown). One having a segregation pattern consistent with a single T-DNA integration locus was chosen for making a line homozygous for the insert. A homozygous transgenic plant line obtained thus is AL66-1. Arabidopsis ecotype Landsberg erecta was infected with Agrobacterium strain AGL1 carrying pMC104, and transformants were selected on phomycin. Three independent transformants were analyzed by RNA gel blot analysis, and the HS104-1 transformant was retained for this study because it showed strong accumulation of the HO transcript after heat shock treatment at 37°C for 2 hr (Figure 2C). Immunobots with anti-HO antibody showed the presence of the HO protein in leaf lysates of this line (data not shown). HS104-1 has at least three T-DNA insertions, as determined by DNA gel blot analysis. It was found that later generations of HS104-1 did not accumulate HO transcripts after heat shock but that HO expression in this line was low and constitutive (data not shown).

GUS Histochemical Staining

Transgenic plants were propagated in sterile agar medium as described previously (Assaad and Signer, 1992). To determine GUS activity, we stained plant tissues with 5-bromo-4-chloro-3-indolyl 3-β-D-glucuronide acid, according to Jefferson (1987). GUS+ sectors appear blue, and their number was determined using a dissecting microscope.

PCR Analysis

An extract containing genomic DNA from leaf tissue was obtained by treatment with 1% cellulase and 0.25% macerozyme in 0.5 M mannitol for 30 min at 30°C, followed by centrifugation to remove cell debris. PCR was performed with the supernatant after digestion with 60 mg/mL proteinase K at 55°C for 1 hr. Primers (1 μM each) were 5'-CAGGTC-AGGTTTTTGACGC-3' (primer 1) and 5'-CAGAAAGTGATGGAGCA-TCA-3' (primer 2). Amplification by Taq DNA polymerase was performed in 1.5 mM MgCl2. Conditions were 30 sec at 94°C, 1 min at 55°C, and 1.5 min at 72°C for 30 cycles. PCR analysis of genomic DNA from calli (Dellaporta et al., 1983) was performed using primers (0.8 μM each) 5'-GATGTGATATCTCCACTGACG-3' (primer 3) and 5'-GTTCTGTTGTCACACAAACGG-3 (primer 4) and Taq DNA polymerase, as suggested by the manufacturer (Perkin-Elmer), with 1.5 mM MgCl2. Conditions for amplification were 3 min at 95°C, followed by 30 cycles starting at 94°C for 30 sec followed by 55°C for 2 min and 72°C for 20 sec.

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