Novel plasmid vectors for homologous transformation of barley (*Hordeum vulgare* L.) with JIP23 cDNA in sense and antisense orientation

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SUMMARY

The most abundant **j**asmonate-**i**nduced **p**rotein (JIP) in barley leaves is a 23 kDa protein (JIP23). Its function, however, is unknown. In order to analyze its function by homologous transformation, new plasmid vectors have been constructed. They carry the cDNA coding for JIP23 in sense or antisense orientation under the control of the *Ubi-1*-promoter as well as the *pat* resistance gene under the control of the *35S* promoter. Barley mesophyll protoplasts were transiently transformed with the sense constructs. PAT activity and immunological detection of JIP23 could be achieved in transformed protoplasts but not in untransformed protoplasts indicating that the construct was active. Thus, these new vectors are suitable for stable transformation of barley. Carrying a **m**ultiple **c**loning **s**ite (MCS), these vectors can be used now in a wide range of transformation of barley.

Key words: *Hordeum vulgare*, jasmonate-induced protein (JIP), transformation

INTRODUCTION

Jasmonic acid (JA) and its methyl ester (JM) collectively named 'jasmonates' are ubiquitously occurring plant hormones (Sembdner and Parthier, 1993, Wasternack and Hause, 2002). Most plants analyzed so far respond to external stimuli such as wounding or other stress by an endogenous rise of jasmonates which is followed by expression of a subset of genes usually induced also upon JA treatment (Wasternack and Hause, 2002). These JA responsive genes code for various groups of proteins including proteinase inhibitors and enzymes of

phytoalexin synthesis. In barley a thionin, a ribosome-inactivating protein and enzymes of JA biosynthesis such as a lipoxygenase (LOX), and the allene oxide synthase (AOS) are among the jasmonate induced proteins (JIPs). For the most abundant JIP of barley leaves, a 23 kDa protein (JIP-23), however, no putative function could be drawn from data base searches. Due to its stress-responsive formation and tissue specific appearance, JIP23 might function as a stress-protective protein (Hause *et al.,* 1996, 1999, Kramell *et al.,* 2000). Furthermore, JIP23 may attribute to the well-known JA-induced down-regulation of photosynthetic genes as shown by heterologous overexpression in tobacco (Görschen *et al.,* 1997). Also in barley, JIP23 may act by "repressing" the amount of photosynthesis-related and nuclear-encoded proteins in cells which have yet to form functional chloroplasts. Here, the homologous overexpression or the antisense repression of JIP23 will allow to elucidate its function in different tissues and developmental stages of the barley plant.

Therefore, novel plasmid vectors have been constructed which contain the cDNA coding for JIP23 in sense or antisense orientation. Transient expression in barley mesophyll protoplast revealed that the constructs were functionally active. Additionally, the introduction of a multiple cloning site (MCS) into the vector plasmid provides easy-to-handle vectors to create further transformation vectors with other cDNAs of interest.

MATERIALS AND METHODS

Plant material and bacterium strain

Barley seedlings of the two-row spring-type cultivar 'Salome' were grown under sterile conditions on hormone-free MS medium for 7 - 10 days.

Plasmids were cloned in the *Escherichia coli* strain XL1-Blue MRF'.

Plasmids, cDNAs and oligonucleotides

pUC18 was used as cloning vector (Norrander *et. al.*, 1983). MCS was composed of the oligonucleotides ***mcs-1*** (5'-TGC CCG GGC ACT AGT ATC GAT CTA GAG CGG CCG CAT GCA-3') and ***mcs-2*** (5'-TGC GGC CGC TCT AGA TCG ATA CTA GTG CCC GGG CAT GCA-3'). ***sb-85*** (5'-AAT TTG CA-3') allowed an *Eco*RI-*Pst*I ligation.

The following plasmids and cDNAs were integrated in the novel constructs:

(i) **pAHC20** containing the *bar* gene under the control of the *Ubi-1* promoter (Christensen and Quail, 1996),

(ii) **pWD26.41** containing the *pat* gene under the control of the *35S* promoter (Dröge *et al.,* 1992), and

(iii) the cDNA coding for JIP23-3 isolated from a cDNA library obtained from barley seedlings 38 h after imbibition (Hause, unpublished).

Molecular biological methods

Basic methods of plasmid manipulation. Competent *E. coli* cells were prepared and transformed according to Inoue *et al.* (1990). Plasmid DNA was isolated by a rapid alkaline extraction and purified according to the manufacturer's instruction (QIAGEN). Restriction analysis, dephosphorylation, ligation and gel electrophoresis were performed as described by Sambrook *et al.* (1989).

Colony hybridization. Basically the method of Sambrook *et al.* (1989) was used. The radioactively labeled DNA probe was prepared from the dephosphorylated oligonucleotide mcs-2 labeled with [γ-^{32}P]ATP by a T4 polynucleotide kinase at 37 °C for 60 min. The prehybridization, hybridization and washing steps were performed according to standard protocols (Sambrook *et al.*, 1989).

Protein extraction and Western-blot analysis. Proteins were extracted from protoplasts according to Meyer *et al.* (1988). Total protein extracts were separated by PAGE in a 12% polyacrylamide gel, and transferred onto nitrocellulose membranes by semi-dry electroblotting.

For immunostaining blots were incubated for 2 h with the rabbit monospecific polyclonal antibody raised against JIP23 (Hause *et al.*, 1996) diluted 1:10000 in 1% BSA/TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% TWEEN 20). After washing, incubation with a goat anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer, Mannheim; diluted 1:2000 in 1% BSA/TBST) was performed for 1 h. Blots were stained with p-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) according to the supplier's protocol.

PAT-assay. Protein extracts of transformed mesophyll protoplasts were incubated with [^{14}C]-acetyl-CoA and phosphinothricin (PPT) followed by thin-layer chromatography (de Block *et al.*, 1987, modified by Schulze, pers. comm.).

Isolation and transformation of barley mesophyll protoplasts

Primary leaves of barley seedlings were cut into pieces of about 2 mm length and were incubated in enzyme solution (4% cellulose and 0.02% pectinase in CPW medium; Frearson et al., 1973) at 25 °C for 15-16 h. Protoplasts were filtered through 56 µm nylon mesh and washed with CPW containing 0.68 M mannitol. Following centrifugation protoplasts were resuspended in 1 ml washing solution (WLW) and were layered onto a 0.6 M sucrose cushion, followed by centrifugation. The green band containing viable protoplasts was collected from the upper phase. Protoplasts were diluted with WLW, sedimented and resuspended in 1 ml WLW. Protoplast number was calculated using a haemocytometer. After sedimentation protoplasts were adjusted to 10^6 per ml with a solution containing of 0.6 mannitol, 15 mM $MgCl_2$ and 0.1% MES. Aliquots of the protoplast suspension (500 µl each) were used for transformation according to Negrutiu *et al.* (1987). Transformed protoplasts were cultured in the culture medium (T/G medium) at a density of 2×10^5 per ml at 25 °C in the dark. Total protein extracts were prepared from protoplasts of day 2, 3 and 6 of cultivation.

RESULTS

Construction of novel vectors containing the JIP23 cDNA

Vectors were designed which carry the barley JIP23 cDNA controlled by the *Ubi-1* promoter and the *pat* resistance gene controlled by the *35S* promoter within the same plasmid. The main steps of cloning were as follows (Fig. 1):

Step 1. The *bar* gene was removed from pAHC20 by partial digestion with *Pst*I. MCS of 35 base pairs, composed of oligonucleotides *mcs-1* and *mcs-2*, was inserted between the *Pst*I sites. As the MCS completes the restriction sites with T instead of G, *Pst*I cannot recognize the original sites any more. Following ligation and transformation, the colony-hybridization and restriction analysis revealed three positive clones. The orientation of the MCS (sense or antisense) was determined by sequencing. For this purpose, MCS-containing fragments of the three clones were inserted into pUC18 by *Eco*RI ligation.

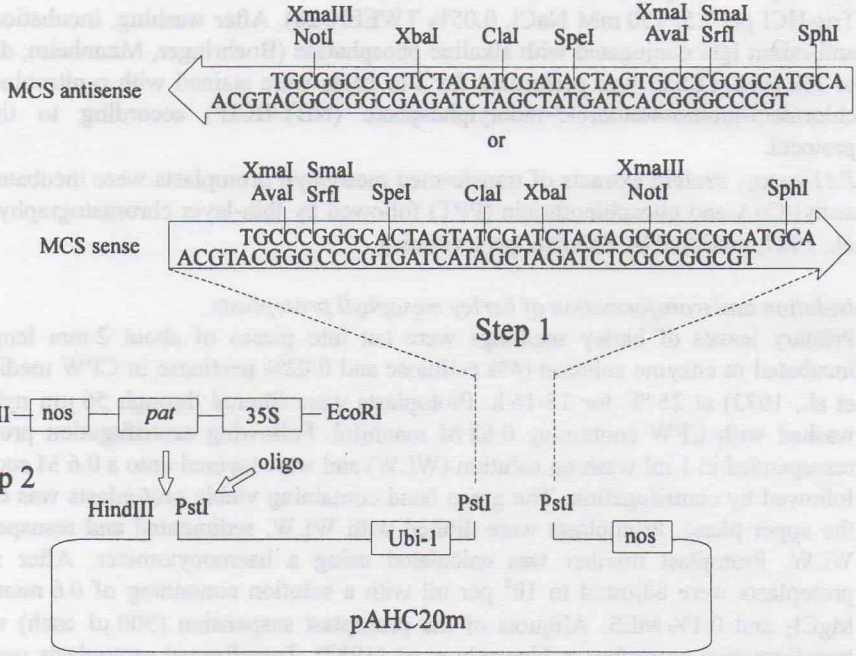

Figure 1. Scheme of construction of the novel vector (cf. text for details)

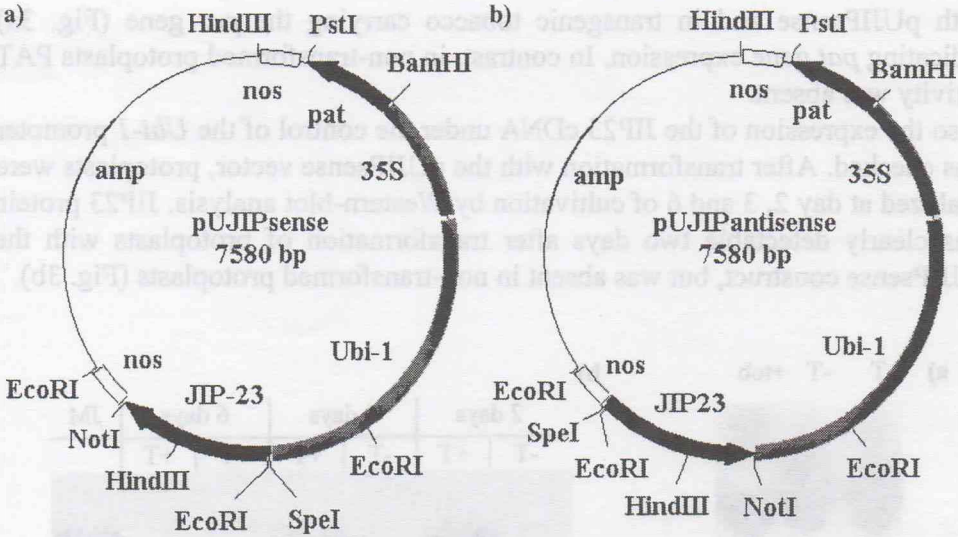

Figure 2. Vectors carrying the JIP23 cDNA in sense (a) and antisense (b) orientation. 35S: CaMV 35S promoter; Ubi-1: maize *ubiquitin* promoter and first intron; JIP23: JIP23-3 cDNA from barley; pat: *pat* gene from *Streptomyces viridochromogenes*; nos: *nopaline synthase* 3' sequence from *Agrobacterium*

Step 2. The modified pAHC20 clones (pAHC20m) containing sense or antisense MCS were cut by *Hin*dIII and *Pst*I and the 1689 bp *Eco*RI-*Hin*dIII fragment of the pWD 26.41 plasmid was inserted using a seven-base pair oligonucleotide to allow *Pst*I-*Eco*RI ligation.

Step 3. Both plasmids containing sense and antisense sequence were cut inside the MCS by *Not*I and *Spe*I. JIP23 cDNA containing terminal *Not*I and *Spe*I restriction sites was inserted into the corresponding vectors.

The vectors containing the JIP23 cDNA in sense and antisense orientation were designated as pUJIPsense and pUJIPantisense, respectively (Fig. 2).

Transient expression of the pat *gene and of the JIP23 cDNA*

In order to test the expression of both sequences, transient transformation of barley mesophyll protoplasts was performed. pUJIPsense was introduced into mesophyll protoplasts of the cultivar 'Salome' by PEG-mediated transformation. After two days of cultivation the PAT activity was determined. PAT activity was clearly detectable in barley mesophyll protoplasts transformed

with pUJIPsense and in transgenic tobacco carrying the *pat* gene (Fig. 3a) indicating *pat* gene expression. In contrast, in non-transformed protoplasts PAT activity was absent.

Also the expression of the JIP23 cDNA under the control of the *Ubi-1* promoter was checked. After transformation with the pUJIPsense vector, protoplasts were analyzed at day 2, 3 and 6 of cultivation by Western-blot analysis. JIP23 protein was clearly detectable two days after transformation of protoplasts with the pUJIPsense construct, but was absent in non-transformed protoplasts (Fig. 3b).

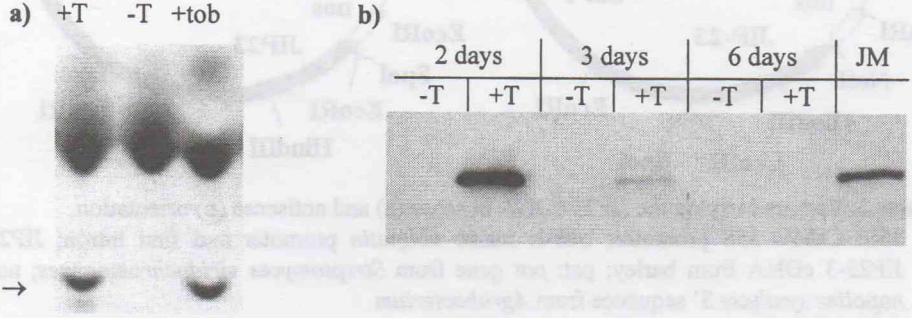

Figure 3. PAT activity (**a**) and JIP23 accumulation (**b**) in transformed barley mesophyll protoplasts.

a) +T: protoplasts transformed with pUJIPsense; -T: non-transformed protoplasts; +tob: transgenic tobacco carrying the *pat* gene; arrow: acetylated L-PPT.

b) JIP23 accumulation in non-transformed (-T) and transformed (+T) protoplasts 2, 3 and 6 days after transformation. For comparison JIP23 was detected in barley leaf segments (LS) treated with JM for 24 h.

DISCUSSION

In order to set up stable transformation of barley via particle bombardment, plasmid vectors have been prepared which contain (i) a cDNA coding for barley JIP23 in sense or antisense orientation under the control of the *Ubi-1* promoter and (ii) the *pat* resistance gene under the control of the *35S* promoter. The use of two different promoters to control a gene of interest and a resistance gene is a repeatedly performed approach to avoid or minimize transcriptional gene silencing (de Wilde *et al.*, 2000). The co-transformation of the expression cassettes (i) *35S::resistance gene* and (ii) *promoter of various origin::transgene of interest* has already been successfully used for the generation of transgenic

barley plants (Jähne et al., 1994, Ritala *et al.*, 1994, Leckband and Lörz, 1998). To date, however, the combination of *35S* and *Ubi-1* promoters controlling different genes in the same plasmid has been reported only for binary vectors used in the *Agrobacterium*-mediated transformation of barley (Horvath *et al.*, 2000, Wang *et al.*, 2001).

The present work describes the construction of a plasmid vector containing both the cDNA of interest and the resistance gene within the same plasmid, both under the control of different promoters. Expression studies in transiently transformed barley protoplasts, done by PAT activity test and immunological detection of JIP23, clearly indicate that both promoters were active and both sequences were expressed, now allowing stable transformation.

Additionally, a MCS was introduced into the basic plasmid in both sense and antisense orientation. This MCS carries restrictions sites for at least ten different restriction enzymes. Therefore, the vector is suitable for cloning other cDNAs for the stable transformation of barley, too. Such a stable transformation with the cDNA encoding JIP23 may now attribute to the functional analysis of this protein.

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