Introduction

Metallothioneins (MTs) are a family of intracellular, cysteine-rich, low molecular mass metalloproteins (M_r < 10 kDa), known for their strong binding capacity of transition metals in metal-thiolate clusters (Coyle et al., 2002). Since the first discovery of MTs in horse kidney (Margoshes and Vallee, 1957), they have been found in most organisms except *Eubacteria* (Capdevila et al., 2005).

MTs typically contain two cysteine-rich domains in the terminal ends of the protein and have historically been divided into three classes according to the number and distribution of cysteines. Class I MTs, found in mammals and vertebrates, are characteristic for their highly conserved cysteine residues, whereas the distribution of cysteines in Class II MTs, present in plants; fungi and invertebrates, are less conserved. Class III MTs include the phytochelatins, which are a family of enzymatically synthesized cysteine-rich peptides. Plant MTs have further been divided into four sub-types based on their amino acid sequences. A unique structural feature of most plant MTs is a long variable cysteine-free spacer region that separates the two cysteine-rich domains (Cobbett and Goldsbrough, 2002; Robinson et al., 1993).

Plant *MT* genes are differentially expressed in a tissue specific manner, as well as being developmentally regulated during plant ontogeny, indicating different functions of the various plant MTs (Heise et al., 2007; Cobbett and Goldsbrough, 2002). Metallothioneins in mammals have been associated with heavy metal detoxification, zinc and copper homeostasis, scavenging of reactive oxygen species, and transfer of essential metals to metalloenzymes, chaperones and transcription factors (Palmiter 1998). Expression of plant *MTs* are regulated by a wide variety of stimuli, such as excessive metal ion concentrations, hormones, heat, cold, drought, salt, wounding and protection of cells against oxidative stress, but there is still limited knowledge available regarding their physiological functions (Akashi et al., 2004; Yuan et al., 2008; Zhou et al., 2005).

Analysis of *cis*-acting responsive elements in plant *MT* promoters has been investigated to a limited extent (Ren and Zhao, 2009). Promoter-GUS experiments in transgenic *Arabidopsis thaliana* with promoters derived from, *e.g.* rice (*Oryza sativa*) and the *Casuarina glauca* tree, indicate that the gene expression of these plant *MTs* is regulated in a complex way due to the many different responsive elements present in their promoter sequences (Ren and Zhao, 2009; Lü et al., 2007; Obertello et al., 2007).

Results and Discussion

To gain more insight into the physiological functions of barley (*Hordeum vulgare*) MTs, we are investigating responses at gene and protein level to metal (Cu, Cd, and Zn) exposures in different tissues. We here report on studies of the regulatory mechanisms controlling the expression of one Class II, type 2 MT from barley. The *HvMT2* promoter has been isolated from the cultivar Golden Promise and subjected to *in silico* analysis of its *cis*-acting responsive elements. We are currently in the process of transforming *Arabidopsis* with promoter-GUS constructs, including truncated versions of the barley *MT* promoter.

Isolation of a barley type 2 MT promoter (HvMT2):

An inexpensive PCR based genome-walking technique modified from Guo and Xiong (2006) and Levano-Garcia and co-workers (2005) was applied for isolation of the *HvMT2* promoter. A set of three gene-specific primers targeting known sequences of the *HvMT2* gene were used to amplify the sequence of interest from genomic barley DNA in combination with four semi-degenerated walker primers with partial degeneracy (designed according to Guo and Xiong, 2006). Two fragments were isolated and sequenced, which allowed the two overlapping sequences to be assembled to one 1468 bp *HvMT2* promoter sequence (unpublished).

Identification of cis-acting elements in the barley MT promoter:
The *HvMT2* promoter sequence that was obtained from the genome-walk was submitted to *in silico* analysis in the PlantCARE and PLACE databases (Lescot et al., 2002; Higo et al., 1999). Table 1 below displays a list of selected *cis*-acting elements, together with the putative function of each element, number of times it occurs in the promoter, as well as a plant reference species in which the element previously has been identified.

Table 1: Selected *cis*-acting responsive elements present in the *HvMT2* promoter

Element	Sequence	Function	Number	*Reference species*
ABRE-like	ACGT(G)	Early response to dehydration	8 (5)	*Arabidopsis*
RYCGAC	RYCGAC	Response to dehydration	3	*Hordeum vulgare*
DRE/CRT	RCCGAC	Response to dehydration, high salt and cold	1	*Oryza sativa*
CRT/DRE	GTCGAT	Response to low temperature	1	*Hordeum vulgare*
LTRE	CCGAC	Response to low temperature	1	*Arabidopsis*
MYC	CANNTG	Response to dehydration, low temperature and abscisic acid	6	*Brassica napus*
MYB1	WAACCA	Response to dehydration or abscisic acid	2	*Arabidopsis*
MYB2	YAACKG	Response to dehydration or abscisic acid	3	*Arabidopsis*
MYB CORE	CNGTTR	Response to dehydration and water stress	2	*Arabidopsis*
ABA	ACGTSSSC	Involved in abscisic acid responsiveness	1	*Triticum aestivum*
ABRE-like	MACGYGB	ABRE related and response to calcium	3	*Arabidopsis*
RY repeat	CATGCA(TG)	Seed specific expression and response to abscisic acid	3 (1)	*Brassica napus*
ARR1	NGATT	Involved in cytokinin responsiveness	5	*Oryza sativa*
CPBCS	TATTAG	Involved in cytokinin responsiveness	1	*Cucumis sativus*
CARE	CAACTC	Involved in gibberellin responsiveness	1	*Oryza sativa*
WRKY	TGAC	Binding site for a transcriptional repressor of gibberellin	3	*Oryza sativa*
CATATG	CATATG	Involved in auxin responsiveness	2	*Glycine max*
GCC BOX	GCCGCC	Response to jasmonic acid, ethylene and pathogens	1	*Lycopersicum*
T/G BOX	AACGTG	Response to jasmocic acid and wounding	1	*esculentum*
AS-1	TGACG	Response to biotic and abiotic stress	1	*Arabidopsis*
BIHD-1	TGTCA	Binding site for a factor involved in disease resistance	4	*Oryza sativa*
W-BOW	TTGAC	Involved in disease resistance	1	*Arabidopsis*
BS1	AGCGGG	*Cis*-acting element for vascular expression	1	*Eucalyptus gunnii*
CACT	YACT	*Cis*-acting element for mesophyll expression	10	*Flaveria trinervia*
RHE	KCACGW	*Cis*-acting element for root hair specific expression	2	*Arabidopsis*
POLLEN1	AGAAA	*Cis*-acting element for pollen specific expression	2	*Lycopersicum esculentum*
CGCG BOX	VCGCGB	Calmodulin binding site	4	*Arabidopsis*
SuRE	GAGAC	Sulphur response element and sulphur deficiency response	2	*Arabidopsis*
ACE	TCY(4-6)GCTG	ACE binding site for activation of *CUP1* and *SOD*	1	*Saccharomyces cerevisae*
CuRE	GTAC	Copper responsive element	2	*Chlamydomonas reinhardtii*
ARE	GTGACNNGC	Antioxidant response element	1	*Casuarina glauca*
TATA box	TATAAA	Core promoter	1	*Pisum sativum*

Note: Symbols used in the sequences are: **B**=G/T/C; **K**=G/T; **N** = A/C/G/T; **R** = A/G; **S**=C/G; **V**=A/C/G; **W**=A/T; **Y** = T/C

According to the responsive elements identified in the *HvMT2* promoter sequence, this barley *MT* gene is potentially subject to a complex regulation by a large number of developmental and environmental factors. There was not identified any core metal responsive elements, which are commonly present in fungal and animal *MT* promoters (Thiele, 1992). Interestingly, however, the barley *HvMT2* promoter contained two copper responsive elements (CuRE) and one ACE-binding site (activation of *CUP1* expression) (Evans et al., 1990; Canessa et al., 2008). ACE is a copper-dependent transcription factor that is known to activate the Cu/Zn superoxide dismutase and *MT* genes (*CUP*) in yeast (Thiele 1992; Gralla et al., 1991). One antioxidant responsive element (ARE) was furthermore identified in the barley promoter.

Comparison of the barley *HvMT2* promoter with a closely related rice *MT* promoter (Ren and Zhao, 2009) indicates that the two *MTs* are regulated differently. According to the number of *cis*-acting elements in the rice promoter, cytokinin is predicted to have the strongest influence on the rice *MT* expression, whereas our *in silico* analysis of the barley *HvMT2* promoter predict it to be more tightly regulated by abscisic acid and drought. The *in silico* analysis of the rice promoter elements was, however, not entirely in accordance with the

subsequent functional analysis in transgenic *Arabidopsis*, as both drought and abscisic acid triggered the promoter to drive a high GUS-expression (Ren and Zhao, 2009).

Heterologous expression of HvMT2 promoter-GUS constructs in Arabidopsis:
The easy transformable plant *Arabidopsis* will be used as a shortcut to investigate the regulation of barley metallothioneins. Hence, we will transform *Arabidopsis* with full length and truncated versions of the *HvMT2* promoter driving GUS expression.

The promoter segments were inserted into a USER-compatible pCAMBIA3300 vector (Nour-Eldin et al., 2006) that previously was modified with an NLS-GFP-GUS-polyA insert from the pBIN19 vector from Chytilova (1999). The truncated versions of the *HvMT2* promoter were designed to exclude specific segments of 100 to 400 bases in a sequential manner from the 5' to the 3'-end, except for the minimal promoter predicted to the last ~100 bases. To investigate if the CuRE and ACE elements are essential for activation of the promoter in response to copper, the two CuREs together with the ACE-binding site was also systematically deleted one by one and a combination thereof. To obtain these truncated promoter versions, USER adapted primers were applied to amplify the specific promoter segments from the full length promoter. The specific promoter fragments were fused with the USER fusion technique, thereby excluding the specific promoter sequences of interest (Geu-Flores et al., 2007). The promoter-GUS constructs were sequenced prior to *Agrobacterium* mediated transformation.

In future work, T_2 or T_3 generations of transformed *Arabidopsis* will be subjected to different treatments, *e.g.* drought, metal stress and hormone treatments, to verify functions of the predicted *cis*-acting responsive elements. Histochemical staining of transgenic *Arabidopsis* plants in different developmental stages will be implemented to reveal the developmental- and organ-specific expression of the barley metallothionein.

Conclusions

The knowledge about the physiological functions of plant metallothioneins is still limited, especially regarding the regulatory mechanisms controlling their expression. We have isolated one type 2 *HvMT* promoter and identified several interesting *cis*-acting responsive elements, which will be functionally analysed in transgenic *Arabidopsis*.

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