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Puroindoline-a and puroindoline-b interact with the *Saccharomyces cerevisiae* plasma membrane through different amino acids present in their tryptophan-rich domain

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Abstract

Puroindolines are two small, basic cysteine-rich proteins isolated from *Triticum aestivum* seeds and characterized by a tryptophan-rich domain. They form the molecular basis of wheat grain hardness and display antimicrobial activity that may contribute to plant defence. Their antimicrobial activity is presumed to be due to their hydrophobic tryptophan-rich domain. However, little is known about their mode of action and there is no *in vivo* evidence that the binding of puroindolines to membranes is mediated by their tryptophan-rich domain. In this study, using a yeast complementation assay, we showed that puroindolines interact with the *Saccharomyces cerevisiae* plasma membrane. By site-directed mutagenesis of their tryptophan-rich domain, we determined that two tryptophan residues (W41 and W44) are mandatory for interaction of puroindoline-a with the yeast membrane whereas interaction of puroindoline-b depends on lysine residues. These results highlight that other residues than tryptophan play a critical role in the interaction of puroindolines with membranes, and probably their affinity for lipids and antimicrobial activities.

Keywords: Membrane interaction; Puroindoline; Tryptophan-rich domain; Yeast complementation assay

1. Introduction

Puroindolines first isolated from *Triticum aestivum* seeds are two basic proteins, puroindoline-a (PIN-a) and puroindoline-b (PIN-b), that contain 10 cysteine residues engaged in five disulfide bonds. Furthermore, both puroindolines are characterized by a unique tryptophan-rich domain; that of PIN-a, WRWWKWWK, is composed of five tryptophan and three basic residues, whereas that of PIN-b, WPTKWWK, is only composed of three tryptophan and two basic residues (Blochet et al., 1993; Gautier et al., 1994). This domain is more or less conserved in puroindoline-related proteins in barley, rye and oat (Darlington et al., 2001; Gautier et al., 2000; Simeone and Lafiandra, 2005; Tanchak et al., 1998).

Puroindolines form the molecular basis of wheat grain hardness, which determines the end-use quality of wheat (reviewed in Morris, 2002). First, a major quantitative trait loci between grain softness and the *PinA* gene locus was identified (Sourdille et al., 1996) followed by the discovery of single-nucleotide polymorphisms (Giroux and Morris, 1997, 1998; Morris et al., 2001). Puroindoline mutations that confer hardness are either a null allele in puroindoline-a (*Pina-D1b*) (Giroux and Morris, 1998) or single nucleotide change in PIN-b (Giroux and Morris, 1997; Lillemo and Morris, 2000; Morris et al., 2001). Two PIN-b mutations, *Pinb-D1b* and *Pinb-D1d*, are localized in the tryptophan-rich domain and resulted in amino acid change G46S and W44R, respectively. In conclusion, when both wild puroindolines are present *T. aestivum* grain texture is soft. In contrast, when either one of the puroindolines is absent or PIN-b altered by mutation, then

Abbreviations: PIN-a, puroindoline-a; PIN-b, puroindoline-b.

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the result is a wheat grain hard texture. Functional evidence was provided by the modification of rice endosperm texture obtained by expressing wheat puroindolines in transgenic rice (Krishnamurthy and Giroux, 2001) and the complementation of hard wheat genotypes obtained by expressing wild-type PIN-b or PIN-a sequences (Beecher et al., 2002; Martin et al., 2006). However, the biological function of puroindolines has not yet been clearly determined, the most favoured hypothesis being a role in plant defence mechanisms. Indeed, these proteins have membranotoxin features due to the presence of a tryptophan-rich domain that displays some similarity with other toxins like tridecapeptide indolicidin, ILPWKWPW WPWRR, isolated from bovine neutrophils (Selsted et al., 1992) or tritrpticin found in porcine white blood cells (Lawyer et al., 1996). Puroindolines display antimicrobial and antibacterial activities *in vitro* (Capparelli et al., 2005; Dubreil et al., 1998) and increase resistance to plant pathogens in transgenic rice (Krishnamurthy et al., 2001) and apple (Faize et al., 2004). In addition, the *PinA* gene is induced by wounding or by a pathogen attack in rice (Evrard et al., 2007).

Puroindolines interact *in vitro* with phospholipids (Wilde et al., 1993), wheat flour polar lipids (Dubreil et al., 1997), and model phospholipid membranes (Le Guernevé et al., 1998). Using synthetic peptides containing the tryptophan-rich domain of puroindolines, it was shown that this domain is involved in the lipid binding (Kooijman et al., 1997) and antimicrobial activity (Jing et al., 2003) of puroindolines. Meanwhile, except some *in vitro* results, there is no experimental evidence that the binding of puroindolines is mediated by their tryptophan-rich domain *in vivo* and most of the data concern PIN-a only. It was therefore of interest to analyse in more detail the binding of puroindolines to a biological membrane and to identify which residues are involved in these interactions to obtain more insight into this mechanism. Here, using a yeast complementation assay based on the activation of the Ras pathway (Aronheim et al., 1994) and site-directed mutagenesis, we identified which residues within the tryptophan-rich domains of PIN-a and PIN-b are involved in their interaction with the yeast plasma membrane.

2. Experimental

2.1. Bacterial and yeast strains

Escherichia coli JM109 (e14(McrA) *recA1 endA1 gyrA96 thi-1 hsdR17(rk− mk+) supE44 relA1* Δ*(lac-proAB)* [*F′ traD36 proAB lacI*^{q} *ZΔM15*]) was used for plasmid propagation and *Saccharomyces cerevisiae* Cdc25 (*Matα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3112 cdc25-2Gal*$^+$; Stratagene) for the yeast complementation assay.

2.2. Plasmid constructions and cloning

pTa31 (GenBank accession number X69913) encoding PIN-a, pTa19B2 (GenBank accession number X69912) encoding PIN-b, and pTaW44R and pTaG46S encoding natural PIN-b mutants cloned, respectively, from *T. aestivum* cv. Creneau and Darius (Gautier, unpublished data) were used as DNA templates. Sequences encoding mature PIN-a and PIN-b were cloned in-frame behind hSos in the pSos plasmid (Stratagene) to create pSos-PIN constructs, and in-frame with the *src* myristoylation signal in the pMyr plasmid (Stratagene) to create pMyr-PIN constructs. A BamHI (pSos cloning) or EcoRI (pMyr cloning) restriction site was created in 5′ of the sense strand primers (P1 and P2, respectively). A SalI restriction site was created in 5′ of the antisense strand M1 primers for cloning either in pSos or pMyr. Sequences of primers (synthesized by Eurogentec) are listed in Table 1.

Table 1
Sequences of PCR primers used to create pSos and pMyr constructs and for site-directed mutagenesis

PIN-a primer[a]	Sequence 5′ → 3′	PIN-b primer[b]	Sequence 5′ → 3′
AP1	*GAATTC*GATGTTGCTGGCGGGG	BP1	*GAATTC*GAAGTTGGCGGAGGAGG
AP2	*GGATCC*CCGATGTTGCTGGCGG	BP2	*GGATCC*CCGAAGTTGGCGGAGG
AM1	*GTCGAC*TCACCAGTAATAGCC	BM1	*GTCGAC*TCATCACCAGTAATAGCC
AP6	ACCGGGCGTGGGGGGAAAGGGGGGAAGGG	BP6	ACCGGGCCCACAAAAGGGGGGAAGGGCGG
AM6	CTTCCCCCCTTTCCCCCCACGCCCGGTG	BM6	GCCCTTCCCCCCTTTAGTGGGCCCGGTG
AP7	ACCGGGCGTGGGGGGAAATGGTGGAAGGG	BP7	ACCGGGGGGACAAAAGGGGGGAAG GGCGG
AM7	CTTCCACCATTTCCCCCCACGCCCGGTG	BM7	GCCCTTCCCCCCTTTAGTCCCCCCGGTG
AP8	ACCTGGCGTTGGTGGAAAGGGGGGAAGGG	BP8	ACCGGGGGGGGGAAAGGGGGGAAGGGCGG
AM8	CTTCCCCCCTTTCCACCAACGCCAGGTG	BM8	GCCCTTCCCCCCTTTCCCCCCCCCCGGTG
AP11	ACCGGGCGTTGGTGGAAATGGTGGAAGGG	BP10	ACCTGGCCCACAGGATGGTGGGGGGGCGG
AM11	CTTCCACCATTTCCACCAACGCCCGGTG	BM10	CCGCCCCCCCCACCATCCTGTGGGCCAGG
AP12	ACCTGGCGTGGGTGGAAATGGTGGAAGGG	BM12	GCCCCCACCACCTCCTGTGGGTCCGGT
AM12	CTTCCACCATTTCCACCCACGCCAGGT	BP12	ACCGGACCCACAGGAGGTGGTGGGGGC
AP13	ACCTGGCGTTGGGGGAAATGGTGGAAGGG		
AM13	CTTCCACCATTTCCCCCAACGCCAGGTG		
AP14	ACCTGGCGTTGGTGGAAAGGGTGGAAGGG		
AM14	CTTCCACCCTTTCCACCAACGCCAGGTG		
AP15	ACCTGGCGTTGGTGGAAATGGGGGAAGGG		
AM15	CTTCCCCCATTTCCACCAACGCCAGGTG		

[a] APx and AMx are, respectively, sense and antisense primers to amplify the PIN-a sequence.
[b] BPx and BMx are, respectively, sense and antisense primers to amplify the PIN-b sequence. Engineered restriction sites are italicized.